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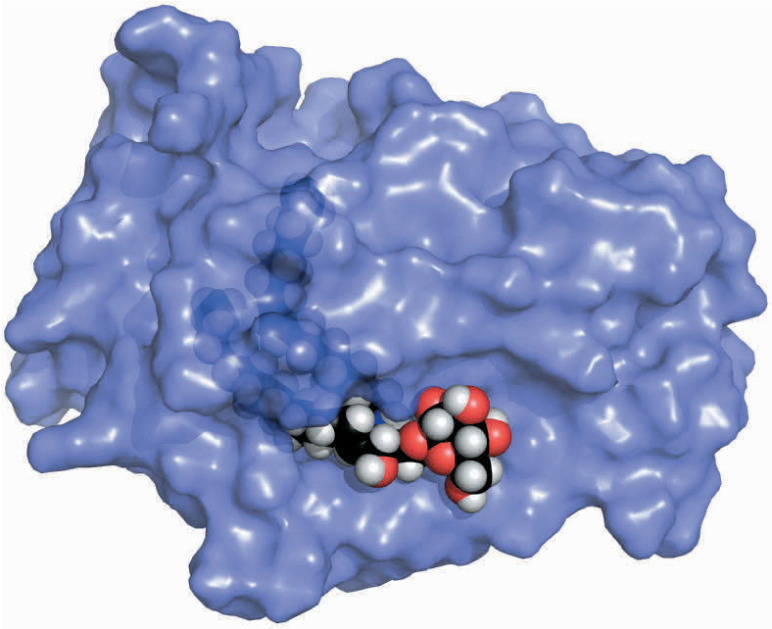
Moving Lipids - Ceramides, Glycosphingolipids and the Glycolipid Transfer Protein



MOVING LIPIDS – CERAMIDES, GLYCOSPHINGOLIPIDS AND THE GLYCOLIPID TRANSFER PROTEIN

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Cover: Surface (van der Waals) rendering of human GLTP complexed with 18:1-GlcCer (PDB:30SK). The image was created in PyMol.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. The publications are referred to in the text by Roman numerals (I – III).

I. Kjellberg MA, Mattjus P (2013) **Glycolipid Transfer Protein Expression Is Affected by Glycosphingolipid Synthesis.** PLoS One.

II. Kjellberg MA, Backman APE, Ohvo-Rekilä H, Mattjus P (2014) **Alternation in the glycolipid transfer protein expression causes changes in the cellular lipidome.** PLoS One.

III. Kjellberg MA, Lönnfors M, Slotte JP, Mattjus P (2015) **Metabolic Conversion of Ceramides in HeLa Cells - A Cholesteryl Phosphocholine Delivery Approach.** PLoS One.

Additional publications not included in this thesis

Nylund M, Kjellberg MA, Molotkovsky JG, Byun HS, Bittman R, Mattjus P (2006) **Molecular features of phospholipids that affect glycolipid transfer protein-mediated galactosylceramide transfer between vesicles.** Biochim Biophys Acta 1758:807–12.

Tuuf J, Kjellberg MA, Molotkovsky JG, Hanada K, Mattjus P (2011) **The intermembrane ceramide transport catalyzed by CERT is sensitive to the lipid environment.** Biochim Biophys Acta 1808:229–35.

CONTRIBUTIONS OF THE AUTHOR

The work for publication I was planned by the author, together with Peter Mattjus. All of the experiments in publication I were performed by the author.

The work for publication II was planned by the author, together with Henna Ohvo-Rekilä and Peter Mattjus. In publication II, the author performed the HPTLC analysis of the radiolabeled GSLs. Together with Anders Backman, the author also performed the immunoblotting and qPCR analysis of the cells used in the various experiments. Anders Backman performed the HPTLC analysis of the radiolabeled phospholipids. Henna Ohvo-Rekilä performed the transfection and cell sorting experiments for the mass spectrometry analysis. The mass spectrometry analysis was performed by Zora Biosciences.

The work for publication III was planned together with Peter Mattjus and Peter Slotte. The author conducted all of the experiments in this work, with the following exceptions: Peter Slotte synthesized the radiolabeled ceramides and Max Lönnfors produced the ceramide/CholPC complexes.

The author of this thesis was the principal author of publications I and III, and wrote publication II together with Peter Mattjus.

ABBREVIATIONS

ACD11	accelerated cell death 11
BFA	brefeldin A
C1P	ceramide-1-phosphate
CerS	ceramide synthase
CERT	ceramide transfer protein
CoA	coenzyme A
COP	coat protein
CPTP	ceramide-1-phosphate transfer protein
ER	endoplasmatic reticulum
FAPP2	phosphoinositol 4-phosphate adaptor protein-2
FFAT	two phenylalanines (FF) in an acidic tract
FRET	fluorescence energy transfer
GalCer	galactosylceramide
Gb3	globotriaosylceramide
GlcCer	glucosylceramide
GlcCerS	glucosylceramide synthase
GLTP	glycolipid transfer protein
GSL	glycosphingolipid
HET-C2	heterokaryon incompatibility protein-C2
HPTLC	high-performance thin-layer chromatography
LacCer	lactosyceramide
LC-MS	liquid chromatography–mass spectrometry
MS	mass spectrometry
LTP	lipid transfer protein
MAM	mitochondria-associated membrane
MCS	membrane contact site
NB-DNJ	N-butyldeoxynojirimycin
ORP	OSBP-related protein
OSBP	oxysterol binding protein
PC	phosphatidylcholine
PDMP	1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH-domain	pleckstrin homology domain
PI	phosphatidylinositol
PITP	phosphatidylinositol transfer protein
PM	plasma membrane
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

PS	phosphatidylserine
qPCR	quantitative real-time polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SM	sphingomyelin
SMase	sphingomyelinase
SNARE	soluble NSF attachment protein receptor
SPR	surface plasmid resonance
START	steroidogenic acute regulatory protein-related lipid transfer
T_m	phase transition temperature
VAP	vesicle-associated membrane protein-associated protein

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ABSTRACT

Lipid movement in cells occurs by a variety of methods. Lipids diffuse freely along the lateral plane of a membrane and can translocate between the lipid leaflets, either spontaneously or with the help of enzymes. Lipid translocation between the different cellular compartments predominantly takes place through vesicular transport. Specialized lipid transport proteins (LTPs) have also emerged as important players in lipid movement, as well as other cellular processes.

In this thesis we have studied the glycolipid transport protein (GLTP), a protein that transports glycosphingolipids (GSLs). While the *in vitro* properties of GLTP have been well characterized, its cell biological role remains elusive. By altering GSL and GLTP levels in cells, we have extracted clues towards the protein's function. Based on the results presented in this thesis and in previous works, we hypothesize that GLTP is involved in the GSL homeostasis in cells. GLTP most likely functions as a transporter or sensor of newly synthesized glucosylceramide (GlcCer), at or near the site of GlcCer synthesis. GLTP also seems to be involved in the synthesis of globotriacylceramide, perhaps in a manner that is similar to that of the four-phosphate adaptor protein 2, another GlcCer-transporting LTP.

Additionally, we have developed and studied a novel method of introducing ceramides to cells, using a solvent-free approach. Ceramides are important lipids that are implicated in several cellular functions. Their role as pro-apoptotic molecules is particularly evident. Ceramides form stable bilayer structures when complexed with cholesterol phosphocholine (CholPC), a large-headgroup sterol. By adding ceramide/CholPC complexes to the growth medium, various chain length ceramides were successfully delivered to cells in culture. The uptake rate was dependent on the chain length of the ceramide, where shorter lipids were internalized more quickly. The rate of uptake also determined how the cells metabolised the ceramides. Faster uptake favored conversion of ceramide to GlcCer, whereas slower delivery resulted mainly in breakdown of the lipid.

1. INTRODUCTION

Lipids are a vast and diverse group of naturally occurring compounds that are generally characterized by their solubility in non-polar solvents. While, technically, a broad array of structurally unrelated molecules fall under this description, lipids are usually defined as fatty acids and their derivatives, as well as substances that are biosynthetically or functionally related to these compounds [1, 2]. Lipids, together with proteins, are major components in the membrane structures that enclose living cells, as well as in the membranes that capsulate the intracellular organelles. The biological membranes function as selectively permeable barriers that define enclosed spaces, in which cells maintain specific biochemical environments. Biological membranes, and the lipids and proteins that they contain, are involved in a variety of essential cellular processes.

In cells, most of the novel synthesis of lipids takes place in the endoplasmatic reticulum (ER), from where the lipids subsequently can be transported to other organelles [3, 4]. Once translocated, the lipids either carry out their designated functions or are further modified to form more complex derivatives. As most lipids are hydrophobic in nature, their transport and translocation within cells rarely occurs as a spontaneous diffusion through the aqueous environment in the cytosol. Instead, cells employ a variety of alternative methods by which they transport lipids. The bulk trafficking of lipids takes place by vesicular transport, where lipids and proteins form cargo-enclosing, water-soluble structures that bud off from membranes and are subsequently directed and transported to their goal destinations [4, 5]. Lipids also diffuse through membranes laterally, or “flip-flop” from one side of a membrane to the other, either spontaneously or with the help of specific translocator enzymes [6, 7]. Lipids can also be transported with the help of lipid transfer proteins (LTPs). The LTPs are a group of specialized proteins that contain hydrophobic cavities in their fully folded structures, to which lipids can bind [8, 9]. LTPs are generally capable of mediating lipid monomer exchange between lipid membranes *in vitro*, however, this is not necessary the case *in vivo*. Some LTPs have been shown to mediate actual lipid transfer in cells, however, sensory and regulatory roles have also been suggested. Overall, the more specific, non-vesicular, protein-mediated method of lipid trafficking is crucial when it comes to maintaining proper cellular lipid homeostasis. An in-depth understanding of the hows and whys of these systems is therefore of great importance.

This thesis will focus on a specific LTP, namely the glycolipid transfer protein (GLTP), as well as its putative lipid interaction partners. GLTP has been shown to bind and mediate the transfer of various glycosphingolipids between model membranes, however, the precise biological role of GLTP is unknown [10]. The work presented in this thesis was performed in an effort to elaborate on the possible biological roles of GLTP, building on the hypothesis that GLTP is involved in the cellular glycosphingolipid homeostasis. In addition to this, a novel, solvent-free method of introducing ceramides to cultured cells has been explored, and its applicability in elucidating GLTP-function is discussed.

Following this introduction, the readers will be presented with a literature overview that lays a foundation for the various themes explored in the works presented in chapter 5. The author has attempted to describe most of the background literature in a brief and concise manner, however, more emphasis will be given to the GLTP-family of proteins, as well as their ligands and possible interaction partners.

2. REVIEW OF THE LITERATURE

2.1 THE BIOMEMBRANE STRUCTURE

Lipids have a variety of important roles in organisms. Their main functions include energy storage (mainly in the form of triacylglycerols and steryl-esters), various signaling events in (or between) cells and serving as structural components in biological membranes. The chemical properties of lipids are essential for the formation of the barrier structures (i.e. biomembranes) that enclose all cells. Segregation of the outside environment from the intracellular space allows for the compartmentalization of the various chemical reactions that occur within cells and are essential for all life. Furthermore, cells also enclose their internal organelle structures with lipid barriers, allowing for a variety of chemical environments to simultaneously co-exist in a cell. These specialized subunits are responsible for distinct functions within the cell, such as sorting and trafficking events, cell respiration and the synthesis and degradation of various biomolecules.

As early as 1925, it was proposed that biomembranes are made up of a bilayer structure, where two leaflets of amphiphilic lipids organize with their polar headgroup regions facing towards the aqueous surroundings [11]. With time, it became apparent that biomembranes also contain protein and carbohydrate components. In 1972, Singer and Nicolson proposed the fluid-mosaic model, which describes the biomembrane as a bilayer of lipids with embedded, integral and peripheral membrane proteins (see **figure 1**) [12]. The fluid-mosaic model described the bilayer structure, more or less, as evenly dispersed in relation to its lipid and protein constituents. Additionally, the membrane lipids were considered to be somewhat static in nature, with more emphasis being given to the protein components, in respect to dynamic membrane functionality. In the decades following the proposal, the model has undergone significant revision. It is now believed that the cellular membranes are highly organized and dynamic assemblies, in terms of both their protein and lipid components.

Over the years, evidence supporting the existence of membrane domains has also emerged [13]. These domains are proposed to be enriched in certain lipid species and proteins that putatively form small, short-lived lateral raft-like regions [14]. Membrane domains are implicated in several of the cellular processes that the membranes function as platforms for. This lateral organization of lipids will be discussed in more detail in section 2.3.2.

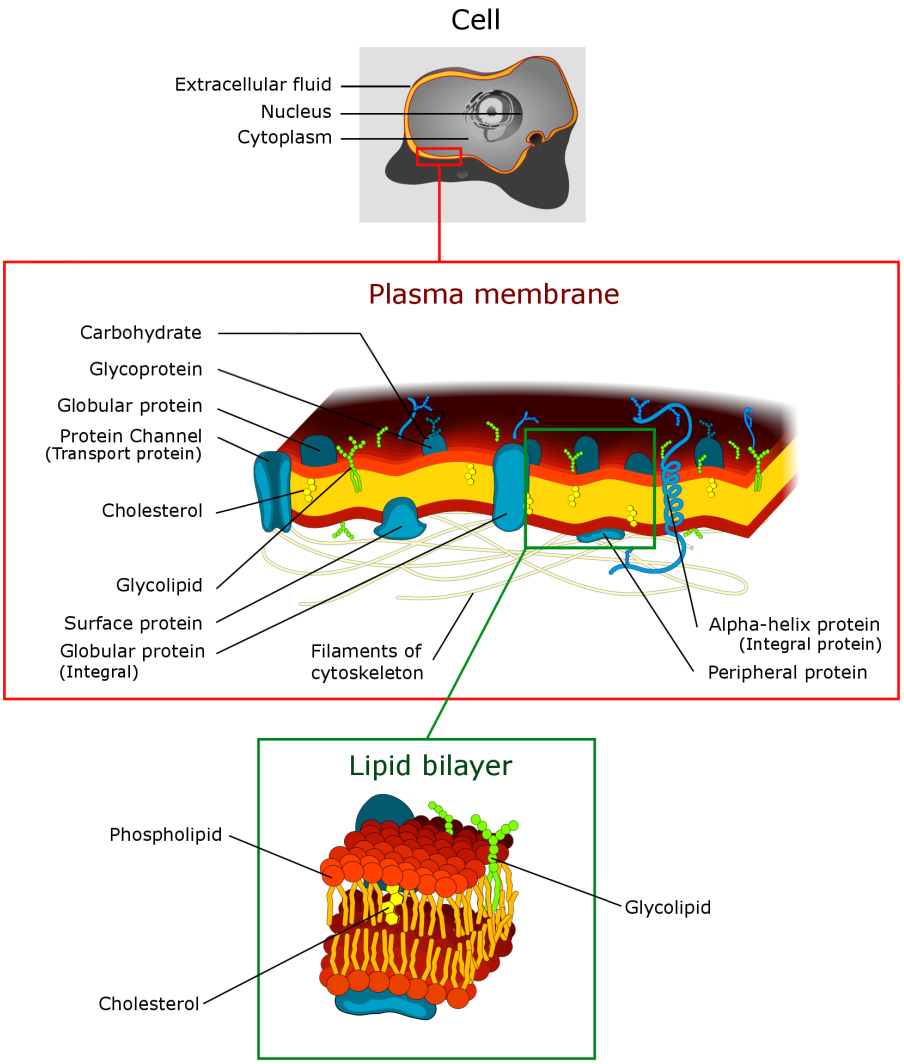

Figure 1. A simplified, schematic representation of the plasma membrane and some of its structural components. The image is adapted from Wikimedia Commons. Attribution: Timothy Gu / CC-BY-SA-3.0

2.2 THE BIOMEMBRANE LIPIDS

Modern mass spectrometry (MS) and bioinformatic methodologies have revealed that cells contain thousands of structurally distinct lipid molecules [2]. Variations in the polar headgroups, as well as the hydrophobic acyl chain regions, give rise to this vast structural diversity. Therefore, in an effort of simplification, lipids are generally divided into several groups, as determined by common structural characteristics. The three main lipid groups that are found in mammalian biomembranes are the glycerophospholipids, the sphingolipids and the sterols. The structures and some of the characteristics of these main membrane lipids will be discussed below.

2.2.1 Glycerophospholipids

The glycerophospholipids are the most abundant lipid species in the mammalian cell, making up 50 – 60 mol% of all the cellular lipids [4]. Their main function is to serve as bulk structural components in biomembranes, however, glycerophospholipids also play a role in cellular signaling events, for example by functioning as precursors for lipid mediators [15]. Glycerophospholipids consist of a glycerol backbone with two fatty acid chains that are esterified to the *sn-1* and *sn-2* hydroxyl groups on the glycerol (**figure 2**). The *sn-3* hydroxyl group is in turn coupled to a polar phosphate [16]. The phosphate can be further modified by the addition of various chemical groups. The most common modification is the addition of a choline, yielding phosphatidylcholine (PC). PC constitutes the bulk of the membrane glycerophospholipids in eukaryotic cells. Natural PC contains fatty acids that are usually between 16 and 18 carbon atoms in length. The *sn*-1 fatty acid is usually saturated, whereas the *sn*-2 fatty acid generally contains 1 to 6 *cis*-double bonds [17]. Other typical glycerophospholipids include phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS).

2.2.2 Sphingolipids

Sphingolipids are not glycerol-based, but instead contain a long-chain aliphatic amino alcohol, a so-called sphingoid base. Sphingolipids generally make up about 10 mol% of the total lipids in mammalian cells [4]. One of the simplest sphingolipids is ceramide, which consists of a sphingosine and a fatty acid, linked together via an amide bond at the C2 position (**figure 2**). Ceramide has a small hydroxyl headgroup linked to the C3 position. In naturally occurring ceramide, the sphingosine base is usually 18 carbons long,

with a *trans*-double bond between carbons 4 and 5, whereas the acyl chain length can vary [18]. Ceramide is a central molecule in the sphingolipid metabolism, as it functions as a precursor for all higher sphingolipids [19]. Ceramide, and its many metabolites, have been implicated in several key cellular functions, including cell growth, signaling, proliferation, differentiation and apoptosis [20–26].

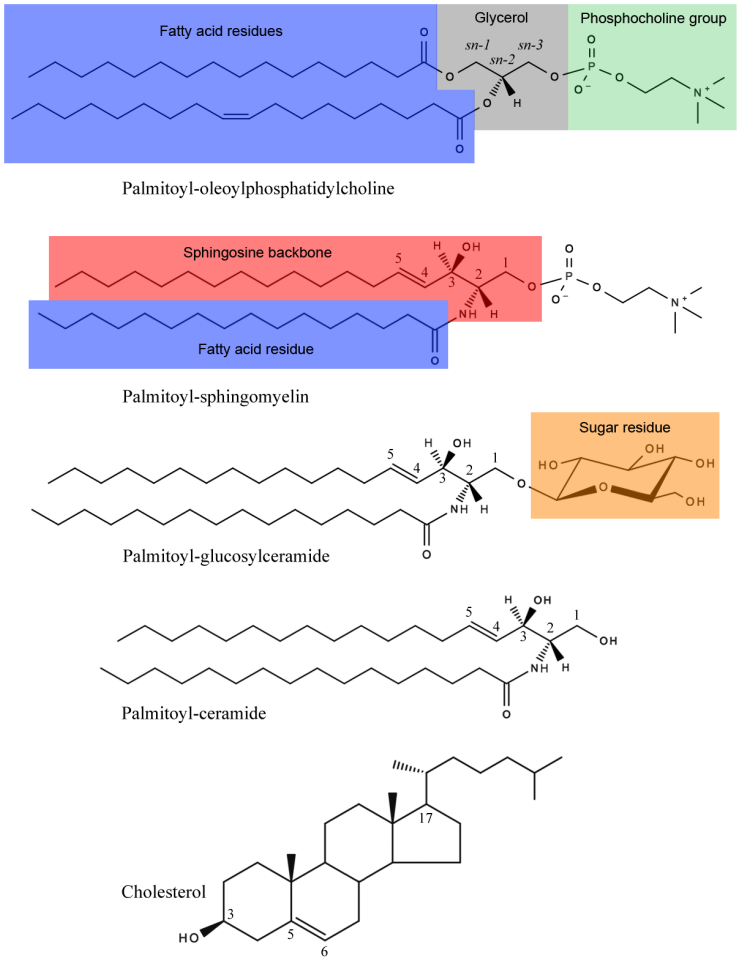

Figure 2. Molecular structures of some common biomembrane lipids. The numbers represent stereospecific numbering (*sn*) or carbon atom positions. The colored boxes indicate some of the building blocks of the various lipids, as described in the text.

Sphingomyelin (SM) is another sphingolipid with key cellular implications. SM is produced from ceramide by the addition of a phosphocholine headgroup. As such, the chain length and saturation of the sphingosine base and acyl chain depend on the precursor ceramide. In mammals, SM is generally the most prevalent of its lipid class and is located mainly to the extracellular leaflet of the plasma membrane (PM) [27]. SM makes up 2 – 15% of the total phospholipid content, depending on tissue type [28]. SM is accumulated in neural tissue, where it plays a role as an insulator in the membranous myelin sheath that surrounds nerve cell axons. Additionally, SM has been implicated in signal transduction, as well as in apoptosis, through a hydrolysis pathway that generates ceramide [29, 30].

Glycosphingolipids (GSLs) are formed in a glycosylation process, where a carbohydrate moiety is attached to a ceramide. The simplest GSLs (the monohexosylceramides) are glucosylceramide (GlcCer) and galactosyl-ceramide (GalCer), which contain a glucose and a galactose molecule, respectively. GlcCer functions as the precursor for most of the higher GSLs found in mammalian cells. The synthesis of higher GSLs takes place by stepwise addition of carbohydrate groups to respective precursors. Some higher GSLs may have up to 20 sugar residues in their headgroups [31]. GSLs are proposed to take part in many cellular processes, such as cell-cell interactions and various signaling events [32]. GalCer, similarly to SM, is a major component of the myelin sheath [33]. GSLs also serve as receptors for various bacterial toxins on the cell surface [32, 34]. As the ceramides and the GSLs are the most relevant lipids in this thesis, they will be discussed in greater detail in sections 2.5 and 2.6.

2.2.3 Sterols

The sterols, with their 4-ring molecular structure, look distinctly different from other biomembrane lipids. Different organisms make use of different sterols. Stigmasterol and sitosterol, for example, are found in plants, whereas ergosterol is found in yeast [35, 36]. The dominant sterol in vertebrates is cholesterol, which makes up roughly 25 mol% of the membrane lipids in cells [37]. Cholesterol contains a hydroxyl group on carbon 3, which acts as its polar headgroup (**figure 2**). Attached to carbon 17 is a short aliphatic chain. Cholesterol cannot form bilayers by itself, however, depending on the surrounding lipids and other environmental factors, it can have a stabilizing effect on the overall membrane fluidity [38]. In addition to being an important constituent in membranes, cholesterol also has regulatory roles in cells. Cholesterol functions as a precursor to vitamin D, the bile acids, as well as the

steroid hormones [39]. Cholesterol is also implicated in cell signaling and membrane trafficking events [40], as well as in many diseases, perhaps most notably atherosclerosis [41].

2.3 LIPID AGGREGATES

Lipids demonstrate amphipathic properties, which means that they have both hydrophobic and hydrophilic parts. Due to these inherent attributes, lipids tend to spontaneously form aggregates with specific conformations when introduced to aqueous environments. The hydrophobic effect is the main driving force behind the formation of a lipid membrane structure. In accordance with the principle of minimum energy, when lipids are dispersed in aqueous solution, maximal entropy is preserved when the hydrophobic parts of the lipids (e.g. the fatty acid chains of glycerophospholipids) arrange to face each other, and the hydrophilic parts (e.g. the phosphate headgroups of glycerophospholipids) face the aqueous environment [42, 43]. Lipids aggregate differently, depending on their physical characteristics, as well as on external factors [43]. Biologically typical lipid assemblies include the nonlamellar micellar, the lamellar, the hexagonal and the inverted hexagonal phases. The lamellar phase describes the bilayer structure, which is the most typical lipid aggregate in a biological system.

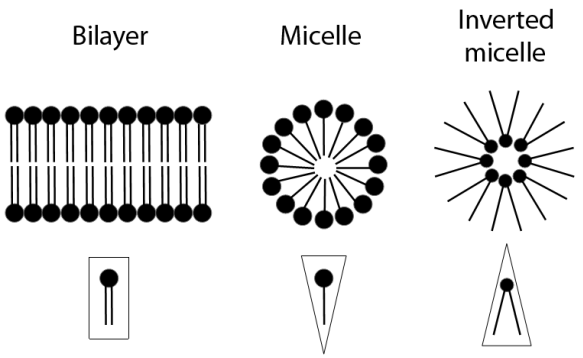

Figure 3. Schematic presentation of some lipid aggregates and how the geometry of the lipids can affect the aggregate shape.

The geometrical shape of the lipid species is the main determinant in the formation of these aggregates (**figure 3**). PC and SM, for example, are cylindrically shaped. The cylindrical shape is the result of the similar physical

cross-sectional area between the polar headgroup and the acyl chains in the lipid. As such, cylindrical lipids form bilayer structures. Geometrically conical lipids, that have either large (e.g. lysolipids) or small (e.g. ceramide) headgroups, in relation to their acyl chain volume, can form micellar and inverted hexagonal structures, respectively [44, 45]. Most membranes in cells contain both cylindrical and conical lipids. Lipids with large headgroups, in relation to their acyl chain volume, will induce a positive curvature on the membrane. Similarly, lipids with smaller headgroups will give rise to a negative membrane curvature. In a biological setting, membrane curvature arises due to the interplay between the variously shaped lipids, as well as certain membrane proteins and membrane-associated proteins [46, 47]. Membrane curvature is implicated in a variety of important cellular processes, perhaps most notably in the budding, fission and fusion of vesicles [47].

2.3.1 Bilayer phases

The bilayer lipid structure can be further divided into distinct phases, which are classified by the varying spatial arrangement and motional freedom of each lipid in the system (**figure 4**) [48]. Both lipid composition (e.g. length and saturation of the acyl chains) and external factors (such as temperature) come into play when defining the bilayer phase. In simple lipid systems, bilayers generally adopt one of two distinct bilayer phases, depending on whether the system exists above or below the main phase transition temperature (T_m) of the particular lipid species used. In temperatures below the T_m, the bilayer exists in a gel-phase (or "solid-ordered" phase), which is characterized by an all *trans*-configuration of the hydrocarbon chains of the lipids. Consequently, the lipids will pack together more tightly, leading to a more rigid membrane structure as well as to a decreased lateral diffusion of the lipids [43, 48, 49].

When the temperature increases over the T_m, the bilayer adopts a liquid-crystalline ("liquid-disordered") phase. This phase is characterized by an increasing amount of *gauche* conformations that are present in the hydrocarbon chains. As a result, the lipids pack together more loosely, the lipids diffuse more readily, the bilayer expands laterally and the thickness of the membrane decreases [43, 48, 49]. When cholesterol is introduced to a lipid bilayer system, a third lamellar phase can be formed, i.e. the "liquid-ordered" phase. In this phase, the lipids are less ordered than in the solid-ordered phase, but also less fluid when compared to the liquid-disordered phase [38]. Here, the acyl chains of the lipids interact with cholesterol to adopt an all *trans*-conformation, similar to that which can be observed in the

solid-ordered phase. In model lipid systems, it has been observed that cholesterol preferentially interacts with sphingolipids [50, 51]. The *umbrella model* describes this preferential interaction as a cause of the sphingolipids shielding cholesterol (using their polar headgroups) from the aqueous environment [52].

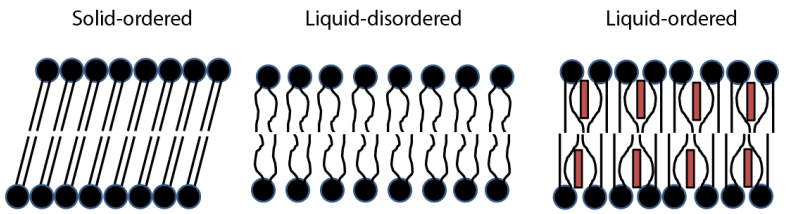

Figure 4. Schematic representation of the solid-ordered, the liquid-disordered and the liquid-ordered bilayer phases. Cholesterol is represented as orange rectangles.

2.3.2 Lateral membrane domains

In 1972, the fluid mosaic model (see section 2.1) described the biomembrane as a generally homogenous mixture of membrane lipids and proteins. The biomembrane contains markedly more lipid species than what is necessary for the formation of a simple bilayer. Therefore, a long-standing question has been what the purpose of such a complex lipid composition could pertain [53]. While the possibility that certain lipids organize as discrete domains in membranes had previously been proposed [54–56], it was not until 1997 that a more refined hypothesis was put forward, suggesting the existence of specific membrane domains (or "lipid rafts") in the plasma membrane (PM) of cells [13]. Lipid rafts (or membrane rafts) are currently defined as "small (10 – 200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" [14]. Many papers have suggested that such lateral domains might play a role in a wide range of biological functions, through activation or recruitment of specific membrane proteins. GPI-anchored proteins and protein tyrosine kinases are examples of enzymes that have been proposed to reside in raft structures [57–60]. The formation of raft structures has also been implicated in vesicular trafficking and in the budding of viruses [61–63].

Generally, research on membrane rafts has focused on the PM, as the outer layer of the PM is enriched in both cholesterol and sphingolipids. However,

reports of raft-like domains existing in other organelles, such as the mitochondria [64, 65], have been presented. Membrane domains have also been suggested to exist in endosomes and lysosomes [66], as well as in the ER [67, 68]. Additionally, it has been suggested that the sorting of lipids destined for PM raft structures already occurs in the *trans*-Golgi network, where sterols and sphingolipids become enriched in secretory vesicles [69]. While the literature describes the properties of ordered domains in model membrane systems comprehensively [70–75], their existence in natural membranes is still lacking in evidence. Initially, the most common methods for detecting rafts in biological systems employed non-ionic detergents and the extraction of so-called detergent resistant membranes, which are enriched in sterols and sphingolipids. These methods, however, are indirect and the results they produce are open to alternative interpretation [53]. It is possible that the detergent resistant membranes are artefacts created by the extraction methods, and therefore would not represent naturally occurring membrane domains. Recently, sophisticated microscopy techniques have become available, allowing for the observation of clustered membrane domains below 200 nm in diameter [76–78]. As membrane rafts are also hypothesised to have short half-lives (estimated to be in the range of 100 nanoseconds or less [79]), their detection is further complicated. The topic of membrane rafts therefore remains somewhat controversial, at least until more compelling evidence for their natural existence is presented.

2.4 LIPID DISTRIBUTION

In biological systems, lipid distribution is generally not even. The types of lipids that are present in different cell types and tissues can vary significantly. In addition, lipid variation occurs between the different cellular organelle membranes, and even between the two leaflets in the membrane bilayer structures. This uneven lipid distribution depends on several factors. First and foremost, different lipids are synthesized at different locations in cells. In addition, lipids can be transported between membrane structures throughout the cell. Since lipids are key players in several essential cellular mechanisms, their synthesis and distribution processes have to be tightly regulated for proper cellular functioning.

2.4.1 Lipid translocation between the membrane leaflets

The lipid asymmetry within and between the two leaflets in a membrane is maintained by a number of processes [4, 37]. Firstly, lipids readily diffuse laterally within the membrane plane. Secondly, lipids can translocate between

the two leaflets of a bilayer membrane, in a spontaneous fashion. The rate at which a spontaneous translocation occurs is dependent mainly on the physical properties of the lipid, as well as its surrounding interaction partners. For lipids with large headgroups, such translocaion is very slow, whereas lipids with small headgroups tend to translocate readily [80–85]. Consequently, cells require specific methods for regulating the transbilayer lipid asymmetry, especially in the case of large-headgroup lipids. This is achieved with the help of translocases (i.e. scramblases, flippases and floppases), which are specialized transmembrane enzymes, capable of translocating different lipid species from one side of the membrane to the other [6, 7]. Flippases and floppases are ATP-dependent enzymes that generally localize to the PM. Flippases catalyse the translocation of specific lipids from the exoplasmic leaflet to the cytoplasmic one, while floppases work in the opposing direction. One of the most studied flippase families is the P4-ATPase family of proteins [86]. Atp8a2, a member of this family, is responsible for the translocation of PS in photoreceptor disc membranes [87]. The floppases include the ATP-binding cassette transporters (ABC-transporters). The ABC-transporters are implicated in multiple drug resistance, as well as several other cellular processes [88]. MRP1 is an ABC-transporter that has been suggested to translocate sphingolipids, including GlcCer and SM, between membrane leaflets [89]. Scramblases are not ATP-dependent. The scramblase catalysed translocation of lipids, however, seems to be calcium-dependent and non-selective [90]. At least 4 human scramblases are expressed in a variety of cells and tissues [91].

2.4.2 Intracellular distribution and metabolism of lipids

The cell is comprised of several organelles with distinct cellular functions. These organelles, like the cell itself, are enclosed by membranes. Lipid distribution between the various organelle membranes is not homogeneous. For the cells to carry out the multitude of biological processes that occur at the various membranes, the correct membrane environments must also be present. The intracellular lipid distribution is a result of how and where the different lipids are synthesized, as well as how the cell transports lipids from their sites of synthesis to their goal destinations. The bulk of the lipid trafficking takes place through vesicular transport pathways, which move cargo between the various membrane compartments [4, 5]. A brief overview of the sites of lipid synthesis, as well as the general lipid distribution between the different organelles will be presented here. As ceramide and the GSLs are the most relevant lipids in this thesis, their synthesis as well as their cellular properties will be discussed more in-depth in sections 2.5 and 2.6.

As mentioned in the introduction, lipids are mostly synthesized at the ER [3]. The ER produces the bulk of the structural phospholipids and cholesterol, as well as ceramide, cholesteryl-esters and triacylglycerol. After synthesis, cholesterol is rapidly transported to other compartments in the cell (mainly the PM), and as such, the ER generally contains only low amounts of this lipid. However, if the cholesterol levels in the PM become elevated, the excess cholesterol can be internalized and transported to the ER for esterification and subsequent storage in lipid droplets [92]. Ceramide is synthesized at (and mainly localized to) the ER [93]. Ceramide is also transported to the Golgi apparatus, i.e. the central cellular sorting compartment, where it is used as a precursor for more complex sphingolipids. A significant level of lipid synthesis takes place at the Golgi, mostly regarding the various sphingolipids. Both SM and the GSLs are mainly produced here [4].

The PM is enriched in sphingolipids and sterols. The majority of the GSLs, as well as SM, localize to the PM. While the PM does not contain enzymes for the autonomous synthesis of its main structural lipids, it still serves as a platform for several metabolic and catabolic lipid events. SM, for example, can be hydrolyzed by a PM-associating sphingomyelinase (SMase), to form ceramide and phosphocholine [30]. In addition, it has been suggested that the PM contains SM synthesizing enzymes [94]. Similar degradation and synthesis events are central in numerous signaling cascades that have been detected to occur at the PM [95].

The earlier endocytic membranes are similar to the PM, but the later endosomes differ significantly. Most notably, cholesterol and PS decreases along the endocytic pathway. The endosomes also contain a system of kinases and phosphatases that produce and hydrolyse phosphorylated PIs (i.e. phosphoinositides) [95]. The phosphoinositides function as important binding-sites for several proteins, allowing for the identification and targeting of specific organelle membranes. Significant lipid synthesis occurs in the mitochondria. In fact, nearly half of the phospholipids found in mitochondria are autonomously synthesized [96]. These phospholipids include phosphatidic acid, PG, cardiolipin and PE. Cardiolipin, which is involved in the mitochondrial energy metabolism, is exclusively found in this organelle.

The breakdown of lipids (as well as that of most other biomolecules) occurs mainly in the lysosomes [97]. Lipids are generally transported to the lysosomes via the endocytic and the autophagocytic pathways. The lysosomes contain several water-soluble hydrolases, which catalyse the degradation of

the biomolecules arriving in the organelle [98]. Consequently, the degraded biomolecules can be re-used by the cell. Lysosomes can also fuse with the PM and discard their content by exocytosis. Lysosomal dysregulation is implicated in several lysosomal storage diseases. The lysosomal storage diseases are caused by defects in the genes that produce the various lysosomal catabolic enzymes, or their activator proteins, leading to the production of inactive or otherwise deregulated variants of said enzymes [99]. As a consequence, the targets of the non-functioning enzymes are accumulated in the lysosomes, oftentimes leading to severe or fatal complications in the patients suffering from the defects. Many sphingolipids are implicated in such diseases. The Gaucher, Krabbe and Sandhoff diseases, for example, result in the lysosomal accumulation of GlcCer, GalCer and GM2-ganglioside, respectively [100].

2.5 CERAMIDE

Ceramide is a central molecule in the sphingolipid metabolism. Ceramide functions as a precursor for all higher sphingolipids and is implicated in a variety of cellular processes, including apoptosis, i.e. programmed cell death [20]. *De novo* ceramide synthesis occurs in a stepwise fashion, on the cytosolic side of the ER, and is catalysed by several membrane bound enzymes (**figure 5**) [101, 102]. The synthesis begins with the condensation of serine and palmitoyl CoA to form 3-dihydrosphinganine, in a reaction that is catalysed by serine palmitoyl transferase [103, 104]. 3-deydrosphingasine is then rapidly reduced to form sphinganine by the enzyme 3-dehydrosphinganine reductase [104]. Sphinganine can now be acylated by different ceramide synthases to form dihydroceramide, which in turn is converted to ceramide by the addition of a *trans*-4 double bond on the sphinganine, by the enzyme dihydroceramide desaturase [105]. Interestingly, the sphingoid base sphingosine is not produced *de novo,* but is only formed through the degradation of ceramide [106]. Sphingosine can be further modified to produce sphingosine-1-phosphate (S1P), which, in contrast to ceramide, is universally involved in survival promotion [107]. At the present, five different mammalian ceramidases have been described, one neutral, one acidic, and three alkaline [108]. Neutral ceramidase activity has been found on the outer leaflet of the PM, whereas the acidic and alkaline ceramidases localize to the lysosomes and the ER/Golgi compartments, respectively. Due to their differing cellular localization, as well as their varying expression patters and substrate specificities, it is likely that each ceramidase is involved in distinct cellular activities [108].

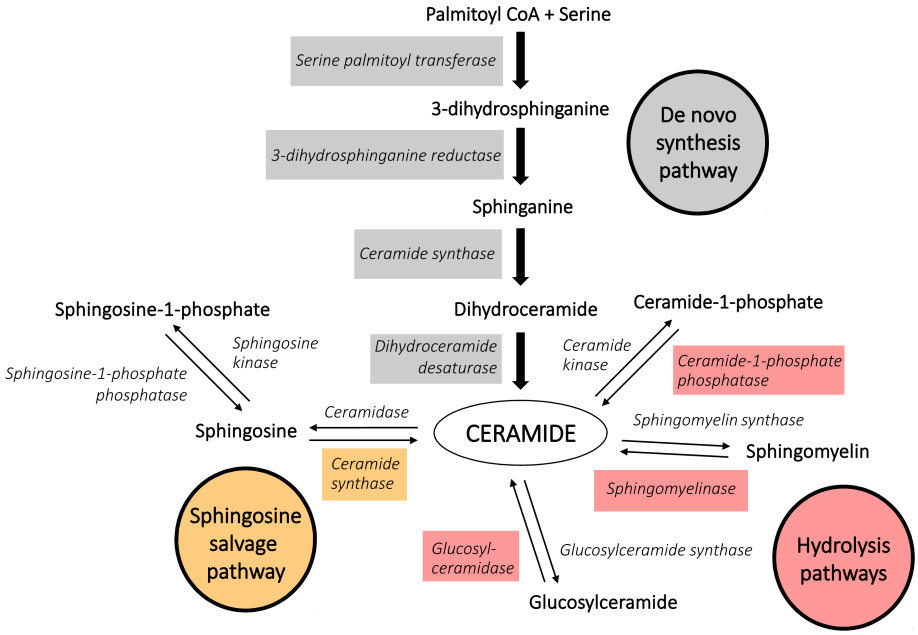

Figure 5. Presentation of the pathways of ceramide synthesis. Synthetic and degrading enzymes are presented in italics and reaction products are presented in bold. The enzymes responsible for ceramide production are color-coded.

In addition to *de novo* synthesis, ceramide can be produced through the sphingosine salvage pathway, where sphingosine that has been released by ceramide degradation is re-used for ceramide synthesis (**figure 5**) [109]. Ceramide can also be generated by hydrolysis of higher sphingolipids, such as ceramide-1-phosphate (C1P), SM and the GSLs. The sphingomyelinase (SMase) pathway describes the synthesis of ceramide though the hydrolysis of SM by various SMases [29, 30]. A non-lysosomal glucosylceramidase has been suggested to produce ceramide through the degradation of GSLs, at or close to the cell surface [110].

2.5.1 The ceramide synthases

The ceramide synthases (CerSs) have emerged as central enzymes in sphingolipid metabolism and biology. As previously mentioned, the CerSs are responsible for the acylation of sphinganine to produce dihydroceramide,

which is subsequently desaturated, yielding ceramide. The CerSs are interesting in the sense that they are not only involved in the *de novo* synthesis of ceramide (as described above), but also in the synthesis of ceramide directly from sphingosine, through the sphingosine salvage pathway [109]. Six distinct CerSs have presently been identified in mammals (CerS1-CerS6), all of which are believed to primarily reside in the ER membranes, where they exhibit their synthesizing activity on the cytosolic side of the membrane [111, 112]. The CerSs might, however, also exhibit more specific localizations. Indeed, there have been reports that show CerS4 and CerS6 activity in the mitochondrial membranes [113–115]. Additionally, CerS1 and CerS6 show perinuclear staining when overexpressed [116, 117]. One of the more notable characteristics of the CerSs, is the ability of the individual isoforms to produce ceramides of particular fatty acyl-chain lengths. The preference of the different CerSs for producing specific acyl-chain length ceramides has been characterized by several studies. All CerSs are acyl CoA dependent, and display preference for certain chain length fatty acyl CoA molecules (**table 1**). Data for CerS acyl CoA specificity is generally derived from *in vitro* analysis as well as siRNA and overexpression experiments.

Table 1. Summarizing table showing acyl CoA preference and tissues of predominant expression of the different CerSs. Information is compiled from various sources (see text).

CerS	Acyl CoA preference	Predominant tissue expression (mouse)
1	C18	Brain, skeletal muscle
2	C22 – C26	Ubiquitous
3	C18 – C24	Testis, prostate, skin
4	C18, C20	Ubiquitous
5	C14, C16, C18	Ubiquitous
6	C14, C16, C18	Ubiquitous

CerS1 mainly produces C18 ceramides, and to a smaller extent also C20 ceramides [118, 119]. CerS2 has a preference for longer-chain fatty acyl CoAs (C22-C26) [120–122]. CerS3 demonstrates a relatively broad fatty acyl CoA substrate preference, producing ceramides with 18-24 carbon acyl chains [123]. Similarly, CerS4 also has a somewhat broader substrate specificity, but demonstrated highest preference for producing medium- to long-chain C18 and C20 ceramides [119, 120]. CerS5 and CerS6 show preference for C14, C16, and C18 acyl CoA [120]. The different CerSs also demonstrate varying expression patterns in different tissues (summarized in [112]). CerS1, for

example is expressed mostly in skeletal muscle and in the brain. CerS2, CerS4, CerS5 and CerS6 are more ubiquitously expressed, whereas CerS3 is mainly expressed in the testis, prostate and skin. Overall, CerS2 is the most widely expressed of the CerSs and is present in most tissues. Additionally, CerS expression undergoes changes during development of various tissues [124, 125]. Taken together, these findings suggest that the different CerSs may be involved in distinct cellular functions, as orchestrated by their preferences for producing ceramides of particular chain lengths.

2.5.2 Ceramide in apoptosis

Numerous studies over the past decades have described ceramides as bioactive lipids that mediate several different cellular functions, such as cell growth, differentiation, and apoptosis [20, 25, 26]. The involvement of ceramide in the cellular apoptotic response is quite evident, and ceramide is implicated both in the extrinsic and intrinsic apoptotic pathways. Cellular ceramide levels are increased in response to a variety of apopototic stimuli. These include environmental stresses, chemotherapeutic agents, as well as the activation of endogenous apoptotic signaling pathways. Apoptotic inducers, such as tumour necrosis factor alpha (TNFα) and Fas ligand (Fas-L) cause rapid accumulation of ceramides in many human cell types [126, 127]. Additionally, extracellular introduction of short-chain (C2- or C6-) ceramides gives rise to apoptotsis [21].

Ceramides function as direct effectors for a variety of enzymes involved in the apoptotic signaling pathways, acting to either suppress or increase their functions. These enzymes include the kinase suppressor of Ras (a scaffolding protein involved in the mammalian mitogen-activated protein kinase [MAPK] pathways), Cathepsin D (a lysosomal aspartic protease), the mixed lineage kinases (a family of MAPK kinase kinases) and protein phosphatase 2 (a serine/threonine phosphatase involved in the regulation of several metabolic processes) [128]. Ceramide has been shown to induce apoptosis via the mitochondrial pathway, through inhibition of the PI 3-kinase/Akt pathway [129, 130]. Additionally, ceramide accumulates in the mitochondria in response to apoptotic signals such as toxins, chemotherapeutic drugs and TNFα [131]. Accumulation of ceramide at the mitochondrial membrane causes permeabilization and consequent release of apoptotic mediators [132]. Interestingly, both ceramide derived from the hydrolysis of SM at the PM, as well as *de novo* synthesized ceramide, seem to be implicated in these responses [133]. It has also been suggested that the turnover of SM at the PM results in the accumulation of ceramide, as well as membrane receptors and

other signaling molecules, in sphingolipid-rich membrane domains that may act as signaling complexes. These membrane platforms would function to potentiate the apoptotic signal transduction, and their formation has been suggested to occur in response to a variety of stressful stimuli [131, 134]. One of the most studied cases involves the clustering and activation of the Fas/CD95 and CD40 cell death receptors in the PM [135–140].

Precursors, derivatives and degradation products of ceramide are also implicated in apoptosis, as well as in cell survival. Sphingosine, like ceramide, accumulates in cells that undergo apoptosis [128]. Unlike ceramide, however, due to being less hydrophobic, sphingosine is not exclusively restricted to membrane fractions. This grants it more flexibility as a second messenger. Sphingosine has many identified signaling-related functions in cells, of which the best studied example is probably the sphingosine-mediated inhibition of protein kinase C [141]. The phosphorylated form of sphingosine (S1P) is, as already mentioned, involved in cell survival mechanisms [142]. In addition, the glycosylated ceramide derivatives, i.e. the GSLs, are also implicated in apoptosis [143]. For example, the ganglioside GD3 has been shown to inhibit survival pathways in the mitochondria [144].

2.5.3 Distinct ceramides with distinct functions

By varying the composition of the hydrophobic tails of the molecule, hundreds of distinct ceramides can theoretically be produced. More than 100 naturally occurring ceramides can be detected using current LC-MS/MS technology [145]. With the discovery and characterization of the various CerSs and with the help of advanced MS-based analysis methods, the ceramides have begun to be defined as a family of distinct lipids with distinct functions, instead of being seen functionally as a single entity. Over the past decades, evidence for particular ceramide species demonstrating specific cellular functions has begun to emerge.

One of the earliest examples of the ceramides structure playing a role in its function comes from the observation that ceramide, but not dihydroceramide, induces apoptosis and other cellular responses [146]. Later studies have revealed that particular chain length ceramides are involved in specific cellular activities. For example, when the B-cell receptor is activated in lymphocytes, a two-stage accumulation of ceramide occurs [147, 148]. The immediate effect shows an increase in C16-ceramide, specifically, whereas in the later stages, C24-ceramide levels are elevated. The later stage accumulation seems to be dependent of caspase activation, in contrast to the

earlier stage. As a whole, this suggests the possibility for distinct ceramides to be implicated in different stages of the apoptotic process. Additionally, specific ceramides have been implicated in diseases, such as cancer. C18:0-ceramide levels have been shown to be selectively down-regulated in head and neck squamous cell carcinomas [149]. Additionally, overexpression of CerS1, which is responsible for the production of C18:0-ceramide, has been shown to increase sensitivity of HEK293 cells to several chemotherapeutic drugs [150]. In these experiments the increased sensitivity was traced back to the selective activation of the p38 MAPK specifically by CerS1, and not by the other CerSs. In colon cancer cells, it was found that CerS6 levels were very low [117] and that, consequently, the C16-ceramide specific activation of the TNF-related apoptosis-inducing ligand (TRAIL) was ineffective. Overexpression of CerS6 in these cells lead to the restoration of TRAIL-induced apoptosis. In contrast, a recent study shows that CerS6-expression is up-regulated in non-small-cell lung cancer [151]. CerS6 is also associated with cell death induced by interleukin-24 in gliablastoma cells [152] as well as with celecoxib-induced cytotoxicity [153]. CerS5 and CerS6 have been shown to be involved in the synthesis of long-chain ceramides through the salvage pathway, in response to UV-radiation in MCF-7 breast cancer cells [154]. In mice, knock out of CerS2 leads to severe liver pathology and neurological degeneration [155, 156].

The many ceramides that exist in eukaryotic cells seem to be involved in a multitude of functions, of which apoptosis is particularly evident. While differing roles for different ceramides have been suggested, overall, this topic remains quite unexplored. The task of elucidating the precise roles of the many ceramides is complicated by the number of existing ceramide species and by the apparent variability concerning their function in different cells and tissues. Additionally, ceramide is a substrate for a multitude of metabolic enzymes in cells (estimated >28 distinct enzymes) [20, 157], which adds to the complexity of the issue. As such, the characterization of the numerous ceramides will be a huge task for future researchers.

2.6 GLYCOSPHINGOLIPIDS

The GSLs, like SM, are derivatives of the ceramides. The GSLs are characterized by their carbohydrate moieties, which can vary in both structure and number. The compositions of the acyl chains also vary, depending on which ceramide species the GSL is based on. The GSLs can be simple or complex, ranging from molecules with a single carbohydrate unit, to lipids with up to 20 attached sugar residues [31]. In cells, the majority of the GSLs

localize to the outer layer of the PM. Similarly to the ceramides, the GSLs are believed to take part in a multitude of cellular functions, including cell-cell interaction, neurodevelopment and cellular signaling events [158–161]. GSLs have also been shown to function as specific receptor molecules for several bacterial toxins and hormones [32, 34]. For example, shiga toxin and cholera toxin bind to globotriacylceramide (Gb3) and ganglioside GM1, respectively, on the PM of cells, prior to their subsequent internalization [162, 163].

2.6.1 GSL synthesis

The simplest GSLs are the glucosylceramides (GlcCer) and the galactosylceramides (GalCer). These GSLs consist of a ceramide base, coupled to a single glucose or galactose molecule through a β-linkage, respectively. GlcCer serves as the basis for most of the higher glycolipids in cells. More than 300 GSLs are believed to use GlcCer as a precursor [164, 165]. The GalCer derivatives are fewer in number, however, GalCer is enriched in a variety of tissues. GalCer biosynthesis is associated with myelination and as such is strongly coupled to glial cells [33]. Consequently, GalCer is enriched in brain tissues, but has also been found to be abundant in epithelia of kidney and intestine [166–168]. The site of GalCer synthesis is subject to some controversy. Both the ER and the Golgi have been suggested as possible sites for the galactosylation process that yields this molecule [169, 170]. In experiments where two different short-chain ceramide variants were used, the galactosylation of 2-hydroxy fatty acid ceramide was shown to occur in the ER, whereas non-hydroxy fatty acid ceramide was galactosylated in the *cis*-Golgi [171]. UDP-galactose:ceramide galactosyltransferase (GalCerS), the enzyme that is responsible for the transfer of galactose to the ceramide backbone, has been shown to reside on the luminal side of the ER [172, 173]. GalCer derivatives include the sulfated GalCer variants (the sulfatides) and the ganglioside GM4 (**figure 6**). Like GalCer, sulfatide is implicated in neuronal function [174]. The remainder of the GSL-producing enzymes are believed to localize to various Golgi compartments [175, 176].

It is generally accepted that GlcCer is synthesized on the cytosolic side of the Golgi, by the GlcCer synthesizing enzyme, UDP-glucose:ceramide glucosyltransferase (GlcCerS) [177–182]. Ceramide destined to become GlcCer is believed to transfer from the ER to the Golgi by vesicular means [183]. The majority of the literature describes GlcCer synthesis to be mainly associated with the *cis*/*medial*-Golgi fractions, however, GlcCerS activity has also been observed in the mitochondria-associated membranes (MAMs) which are a subdomain of the ER, as well as in the *trans*-Golgi regions [179, 184, 185].

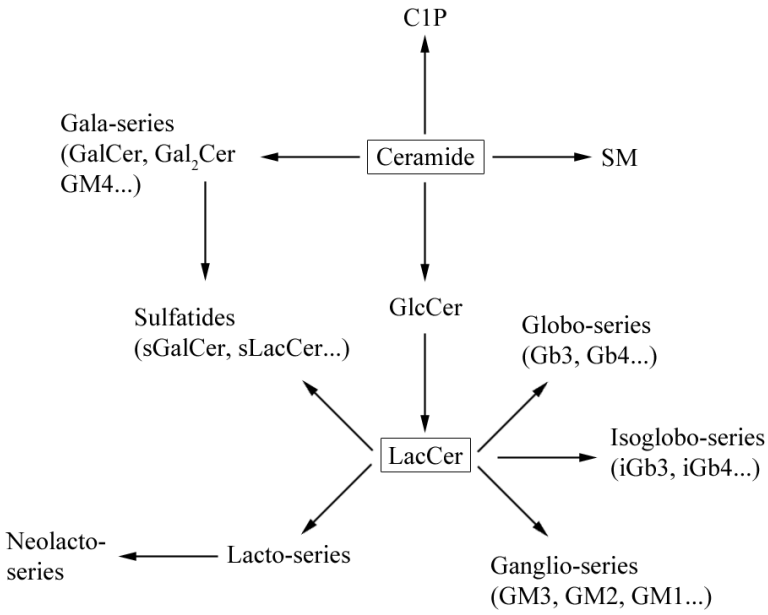

Figure 6. Simplified depiction of the higher sphingolipid synthetic pathways. Ceramide and LacCer (boxed) function as branching points in the synthetic pathways.

The synthesis of the more complex GSLs takes place by a sequential addition of different sugar residues to respective precursor sphingolipids, within the lumen of the Golgi. Distinct enzymes transfer the carbohydrate moiety from a sugar nucleotide (e.g. UDP-glucose, UDP-galactose and CMP-sialic acid) to a specific position on the precursor molecule. Since GlcCer is synthesized on the cytosolic side of the Golgi, it must translocate to the luminal leaflet by some means, prior to its modification to higher GSLs. Due to its large polar headgroup, it is unlikely that GlcCer would flip-flop spontaneously. The translocation is therefore believed to be catalysed enzymatically. Indeed, fluorescent short-chain GlcCer analogues have been shown to cross the Golgi membrane, in a process that is sensitive to ABCB1 multidrug transporter inhibitors [184, 186, 187]. However, the aforesaid GlcCer analogues exhibit significantly differing membrane properties when compared to their natural counterparts, and the data collected may therefore not accurately represent the natural processes [188, 189]. Following its translocation to the luminal side of the Golgi, GlcCer is galactosylated to form lactosylceramide (LacCer) [190,

191]. Once produced, LacCer is not translocated back to the cytosolic side of the membrane [192, 193]. Instead, LacCer functions as a branching point in the synthesis of the higher GSLs that occurs in the *trans*-Golgi network. These higher GSLs can be characterized by their root structures and comprise the globo-, isoglobo-, lacto-, neolacto-, and ganglio-series of GSLs (**figure 6**) [175]. According to the LIPID MAPS Structure Database, some 400 glycosylated sphingolipids have been identified, varying in both headgroup and acyl chain composition [2].

2.6.2 GSL function

While the GSLs have in some cases been shown to be dispensable when it comes to cell survival [194], overall they are essential for normal function in multicellular organisms. Removal of specific GSL-synthetic pathways oftentimes yields only mild phenotypes. This is generally attributed to the compensatory abilities of the remaining GSLs, or alternatively, to the specificity of the ablated GSLs in performing very particular functions [195, 196]. The function of the GSLs has been studied using several different approaches. Many GSLs have been shown to be involved in developmental processes in mammals. Most of the knowledge pertaining to this comes from mouse studies, employing genetic regulation of the various enzymes involved in GSL metabolism. Removal of the GlcCer synthesizing enzyme in mice leads to embryonic lethality during gastrulation [197]. Similarly, removal of the LacCer synthesizing enzyme leads to a termination of embryonic development [198]. Based on several studies, elimination of ganglioside synthesis seems to have less deleterious effects, however, the various gangliosides appear to be involved in the proper development of many neuronal functions. Loss of GM3 synthesis has, for example, been associated with enhanced insulin sensitivity, impaired neuropsychological behaviour and hearing loss [199–201]. Removal of the GA2/GM2/GD2 synthase results in male infertility, axonal degeneration, myelination defects, motor deficits and Parkinsonism [202–206]. Removal of GalCer synthase similarly induces neuronal changes, most probably due to GalCer being a major component of the myelin sheaths surrounding the axons [33]. GSLs are also associated with several diseases in humans. As mentioned in section 2.4.2, some rare lysosomal defects can result in the abnormal accumulation of specific GSLs, resulting in severe and oftentimes lethal conditions [99]. Additionally, GSLs have been implicated in many cancers. A large number of tumour-specific antigens have been identified as GSLs, and changes in GSL expression patterns are associated with several different cancer types [161, 207].

As the GSLs are mainly located to the outer layer of the PM, many of their proposed functions take place there. As mentioned previously, certain GSLs function as binding sites for bacterial toxins, facilitating their entry into the cell [32, 34]. Additionally, GSLs at the PM are implicated in cell-cell recognition and adhesion, as well as in signaling towards the cell interior following these events [158, 159, 161]. A number of GSLs have been found to interact with membrane-located proteins and to modulate their function [208–212]. The GM3-mediated inhibition of the epidermal growth factor receptor at the PM, and the subsequent inhibition of cell growth, is perhaps one of the best-characterized examples of such a GSL-protein interaction [208]. One unique feature of the GSLs is their ability to self-aggregate and cluster in membranes. While the existence of lateral, sphingolipid-enriched domains is subject to some controversy (see section 2.3.2), these domains have been suggested to serve as platforms for a variety of cellular processes [161].

GSLs have also been shown to play roles in the internal organelle membranes of cells. For example, a specific function for GlcCer has been shown in the Golgi apparatus in melanocytes. While mice with null-alleles for GlcCerS are not viable [197], a mutant mouse melanoma cell line that is unable to produce GlcCer is proliferative [194]. An interesting observation regarding these cells is that despite containing all the enzymes required for pigmentation, they still lack the ability to produce melanin [213]. It turns out that GlcCer is required for the transport of tyrosinase, the first and rate-limiting enzyme in the pigmentation process, from the Golgi to the melansomes. Lack of GlcCer leads to tyrosinase accumulation in the Golgi membranes and loss of pigmentation. The exact method by which GlcCer facilitates tyrosinase translocation is unknown, however, it is postulated that GlcCer is involved in the budding of transporter vesicles destined for the melanosomes.

2.7 INTRACELLULAR LIPID TRANSPORT

As mentioned in previous sections, lipids are transported within cells by various means. Given their hydrophobic nature, lipid transport generally does not occur by their free diffusion through aqueous environments, unless the lipid in question is particularly water-soluble. Consequently, the cell utilizes alternative methods for the intracellular transportation of lipids (and other cargo). These methods will be briefly discussed below.

2.7.1 Vesicular transport

Eukaryotic cells are characterized by their endomembranes: a system of organelle membranes that are connected to each other by vesicular transport along the secretory and endocytic pathways. Vesicular transport is the major method by which cells traffic cargo in and out of the cell, as well as between various membrane-bound compartments within the cell. The bulk transfer of lipids also occurs by this method [4, 5]. Cellular vesicles are bilayer constructs consisting of lipids and proteins and are produced in tightly regulated budding processes at the membranes of the various organelles. Vesicles are constantly budding from and fusing with organelle membranes, especially at the PM, the ER and the various Golgi cisternae. The budding process is initiated by the accumulation of certain lipids and proteins at the membrane surface. As mentioned in section 2.3, the clustering of conically shaped lipids (and shaped transmembrane proteins and membrane-associated proteins) impart membrane curvature and helps initiate vesicle budding [47].

The cytoplasmic surface of the forming vesicle is coated with specialized coating proteins, which are recruited from the cytosol [214]. The coating proteins are the main driving force behind the distortion of the membrane, and act to stabilize the shape of the curving membrane. The coat proteins, together with transmembrane cargo receptors, are also used to recruit specific cargo to the site of vesicle formation. The formation process is completed by the budding membrane being "pinched off" by enzymatic means, which leads to the cargo being engulfed within the vesicle. Over short distances, vesicles travel by diffusion. Transport over longer distances, such as when vesicles move from the Golgi to the PM, is facilitated by motor proteins that move the vesicles along the cytoskeletal filament. As the vesicle fuses with its target, the cargo within the vesicle is released into the opposing side of the membrane, and the lipid and protein constituents of the vesicle bilayer are combined with the target membrane. Prior to fusion, the protein coat surrounding the vesicle is disassembled.

Several types of coated vesicles exist. The first to be characterized were the clathrin coated vesicles, which function to internalize extracellular material at the PM, as well as to transfer cargo from the *trans*-Golgi to the lysosomes (**figure 7**) [215]. In addition, clathrin-independent internalization has been shown to occur [216]. For example, caveolar endocytosis involves the caveolin coat and is utilized in the internalization of GSLs and some viruses. The COP (coating protein) -coated vesicles are responsible for the trafficking of vesicles between the ER and the various Golgi cisternae (**figure 7**). COPII-

coated vesicles bud at the ER and carry their cargo towards the Golgi complex, where the cargo is sorted and modified according to cellular needs. In contrast, COPI-coated vesicles traverse in the opposite direction, in retrograde transfer from the Golgi to the ER, as well as from later to earlier Golgi cisternae [217]. The method by which cargo is transported through the Golgi cisternae is not fully understood, but several models have been proposed [218]. All eukaryotic cells have constitutive secretory vesicles, which continuously bud from the *trans*-Golgi network and travel to the PM. This process delivers lipids and proteins to the cell surface, and allows for the release of cargo to the outside of the cell by the process of secretion [219]. Cells with dedicated secretory functions (such as endocrine cells) can store secretory vesicles in the cytoplasm, until the cell receives an appropriate signal and the vesicles fuse with the PM and release their content into the extracellular environment.

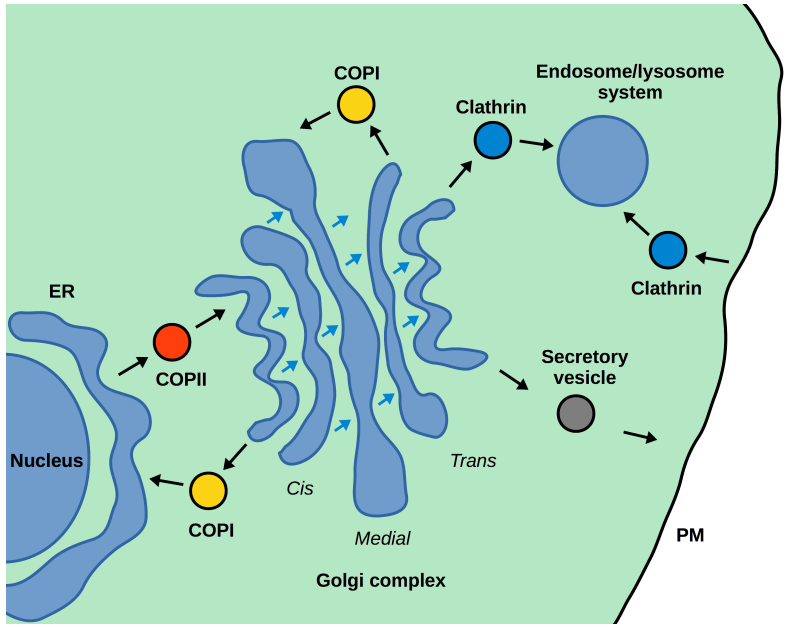

Figure 7. Simplified presentation of known vesicular transport routes along the endomembrane system in a cell. The COPI-, COPII- and clathrin-coated vesicles, as well as the secretory vesicles, are depicted. The blue arrows indicate cargo moving along the various Golgi stacks.

Proper cellular function requires for transport vesicles to be selecively targeted to their goal destination. This recognition and initial attachment (i.e. tethering) is believed to occur through the interplay of so-called tethering factors on the target membranes, and specific targeting GTPases (Rabs) on the vesicles surface [220]. The subsequent fusion of the vesicle to the target membrane is believed to be facilitated through the pairing of SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), where a SNARE on the transport vesicle (v-SNARE) pairs with its complementary target membrane SNARE (t-SNARE) [221].

2.7.2 Lipid transfer proteins

In addition to the vesicular transport mechanisms, LTP-mediated lipid transfer has emerged as an important method of lipid translocation in cells [8, 9, 222]. While many proteins have the capacity to interact with lipids, the LTPs are specifically defined as proteins that facilitate and/or accelerate a lipid exchange between membranes *in vitro* [9]. Additionally, LTPs are characterized by the presence of a lipid-binding hydrophobic pocket in their structures and they generally show specificity for a particular lipid species. Several LTPs have been discovered in eukaryotes, plants and in bacteria. Many of these LTPs have been cloned and crystallized, allowing for their 3D-structures to be determined. LTPs can be divided into several protein families, based on the sequence and structure similarity of their lipid binding domains. In eukaryotes, these families include the SEC14 family, the SCP-2 family (sterol carrier protein 2), the plant nonspecific lipid transfer proteins (plant nsLTPs), the PITPs (phosphatidylinositol transfer proteins), the STARTs (steroidogenic acute regulatory protein-related lipid transfer proteins), the GLTPs (glycolipid transfer proteins) and the ORPs (oxysterol-binding protein-related proteins), and are summarized in **table 2** [223–228]. The different LTP families will be discussed below briefly.

The PITPs are a family of proteins that transfer PCs and PIs between membranes *in vitro* [229]. In mammals, the PITPs consist of at least five members: PITPα, PIPTβ, RdgBβ, Nir2 and Nir3. The first two are referred to as Class I PITPs, whereas the last three are Class II PITPs [9]. PITPs are implicated in phosphoinositide metabolism and related cellular processes, such as phospholipase C signaling and exocytosis [230, 231]. The members of the SEC14 family also transfer PCs and PIs, however, SEC14s do not share sequence or structure homology with PITPs [232]. The prototype SEC14 family member is the yeast protein Sec14p, which is strongly implicated in PI and PC metabolism in yeast cells [223].

The STARTs are a protein family characterized by their START domains. Various START family members bind and transfer different lipid species, such as cholesterol, ceramide and PC [233]. The archetypical START domain is found in the steroidogenic acute regulatory protein (StAR), which mediates cholesterol transfer in the mitochondria [234].

Table 2. Summarizing table of the different LTP families.

LTP family name	Lipid specificity (*in vitro*)
Phosphoinositol transfer proteins (PITP)	PIs and PCs
SEC14	PIs and PCs
Steroidogenic acute regulatory protein-related lipid transfer proteins (START)	Sterols, ceramide and phospholipids
Oxysterol-binding protein-related proteins (ORP)	Sterols, phospholipids
Glycolipid transfer proteins (GLTP)	Glycolipids, sphingolipids
Sterol carrier protein 2 (SCP-2/nsLTP)	Nonspecific
Plant nonspecific lipid transfer proteins (plant nsLTP)	Nonspecific

The mammalian sterol carrier protein 2 (SCP-2) transfers cholesterol, most phospholipids, glycolipids, fatty acids and fatty acyl CoA and is considered a nsLTP [235–237]. The gene for mammalian SCP-2 encodes two proteins: the aforementioned SCP-2, as well as a protein called SCPx [228]. SCPx has been shown to play a crucial role in the peroxisomal oxidation of branched-chain fatty acids [238]. SCP-2 is implicated in cholesterol biosynthesis, uptake, oxidation, esterification and recycling [239–241].

SCP-2 should not, however, be confused with the plant nsLTPs, which are structurally distinct from SCP-2 and are considered as a separate family of proteins [242]. Plant nsLTPs are involved in a variety of functions, including stabilization of membranes, cell wall organization, and signal transduction [242]. The plant nsLTPs can bind a variety of lipids, including fatty acids, phospholipids, glycolipids and prostaglandin B_2 [243].

The ORPs are present in a wide range of eukaryotes. In mammals, these proteins are encoded by 12 genes, yielding several more ORP products due to

splice variation and different promotors [228]. The ORPs contain a beta-barrel-like ligand-binding domain that is able to bind sterols, and in some cases, phospholipids [244]. While ORPs have been implicated in cellular sterol transport, conclusive evidence for this function has not emerged [227]. ORPs have been shown to be involved in sensory and signaling events in cells. For example, the oxysterol-binding protein (OSBP, the founder of the ORP family), acts as a sterol-sensing scaffolding factor and regulates the dephosphorylation of the extracellular signal activated kinases [245].

The lipid specificities and transferring capabilities of the various LTPs have generally been determined by *in vitro* lipid-transfer assays, using either radio- or fluorescently labeled lipids [9, 10]. While the LTPs are named based on their proficiency for transferring specific lipids *in vitro*, it is not always clear whether or not their *in vitro* transfer capabilities reflect their physiological functions. This is a recurring problem within the field of LTP-research, which stems from the lack of methods for direct observation of *in vivo* LTP-mediated lipid transfer. As mentioned above, alternative roles for various LTPs have been proposed, such as signaling, sensory or reporter functions [8, 9]. For example, Nir2 has been suggested to sense and regulate the levels of PC in the Golgi membranes [246]. Nevertheless, some LTPs are believed to be *de facto* lipid transporters. For example, the ceramide transfer protein (CERT) is a START family protein which has been shown to catalyse the transfer of ceramide from the ER to the site of SM synthesis at the late Golgi [247, 248]. Another *de facto* LTP is the four-phosphate adaptor protein 2 (FAPP2), a GLTP-family protein which is required for the transfer of GlcCer to the late Golgi compartment and for the subsequent modification of GlcCer to higher GSLs [183, 249]. The remaining sections of this literature overview will focus on the GLTP family of LTPs, and its founding member, the mammalian GLTP.

2.7.3 The glycolipid transfer protein

GLTP was first described by Metz and Radin in the early 1980s, who demonstrated that a protein residing in the cytosolic fraction of bovine spleen was able to transfer GlcCer between rat erythrocytes and liposomes *in vitro* [250]. Since its initial discovery, GLTP and several of its homologs have been found in a wide range of tissues and organisms, including mammals, plants and yeast [183, 251–254]. The human *gltp* gene is located on chromosome 12 at locus 12q24.11. An additional, transcriptionally silent pseudogene for GLTP has been found on chromosome 11 [255]. GLTP is a small (~24 kDa, 209 amino acids) soluble protein, which resides in the cytosol [256] and has

been shown to bind and transfer a wide range of GSLs between model membranes [10]. Over the years, several of GLTP's biochemical characteristics have been determined. These include, among others, the structural determinants that allow for GLTP to interact with membranes and bind lipids. Despite being biochemically well characterized, the cellular role of GLTP remains elusive. The fact that GLTP is widely expressed and highly conserved between many different organisms suggests that it plays an important biological function.

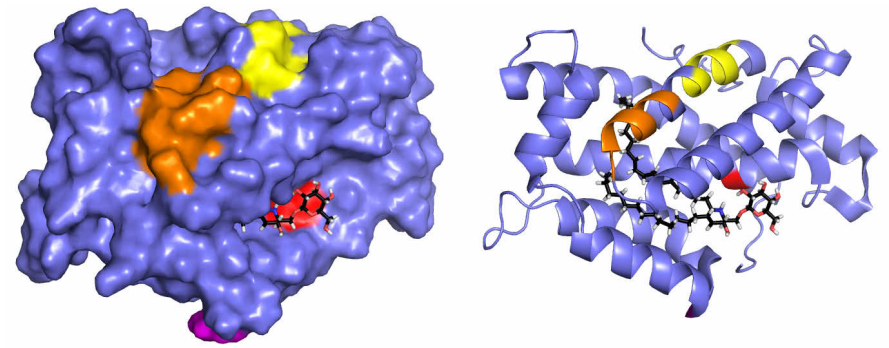

Figure 8. Surface (van der Waals) rendering (left) and a cartoon representation (right) of a crystal structure of human GLTP, complexed with GlcCer containing an 18:1 acyl chain (stick cartoon). The up- and the down-stream FFAT-like motifs are seen as yellow and orange, respectively. W96 and W142 are represented in red and magneta, respectively. The image was created in PyMol using a pdb-file (3S0K) obtained from the RCSB protein data bank.

Initial studies on GLTP function were hampered by labor-intensive purification processes, which resulted in low yields of purified GLTP [257]. In the early 2000s, the successful molecular cloning of the *gltp* gene allowed for rapid production of various mammalian GLTPs, and ushered in a new era of GLTP-research [251, 258]. Large quantities of pure GLTP allowed for the crystallization of the protein and the subsequent determination of the GLTP 3D-structure [259–261]. To date, several crystal structures of GLTP are available in the Protein Data Bank, both in the ligand-free apo-form as well as complexed with various GSLs. The GLTP-fold is unique from many other LTPs in that it consists of an all alpha-helical structure, arranged as two orthogonal layers that “sandwich” a single glycolipid (**figure 8**). The unique structure and the specificity for glycolipids eventually lead to GLTP becoming the founding member of a new superfamily of proteins [262, 263].

Molecular mapping studies on the glycolipid-binding site of GLTP show thatthree distinct regions on the GSLs are involved in the formation of the GLTP-GSL complex: the sugar headgroup, the amide-linkage on the ceramide backbone and the hydrophobic acyl chains [260–262]. The involvement of these regions is evidenced by studies showing that GLTP-mediated GSL transfer is unaffected by free sugars, and that GLTP exhibits poor interaction with mono-chain glycolipids [264, 265]. In mammalian GLTPs, the anchoring of the glycolipid sugar headgroup is stabilized by several hydrogen-bonds, involving the amino acid residues aspartic acid 48, asparagine 52, lysine 55 and tyrosine 207, whereas histidine 140 and aspartic acid 48 are involved in the recognition and binding of the amide on the ceramide backbone [226, 261]. Tryptophan 96 (W96) is key in helping to orient the hydroxyl group on the first sugar moiety of the glycolipid substrate for proper hydrogen bonding. Consequently, W96 has been shown to be essential for the glycolipid transfer activity of GLTP [261, 265, 266]. A point-mutant of GLTP, that replaces W96 with alanine (W96A), exhibits an almost complete lack of transfer activity.

The membrane interaction region of GLTP is composed of several non-polar amino acid residues that form a ring around the opening to the hydrophobic cavity [226]. These residues are typical for membrane interaction, but are organized spatially in a way that differs from certain other membrane-lipid binding motifs, such as the C1 and C2 domains found in phospholipases and protein kinases, as well the pleckstrin homology, FYVE and phox domains that bind to the PIs [267–269]. These particular lipid-binding domains bind to the lipid headgroup while the lipid remains bound to the membrane, whereas GLTP embeds the lipid hydrophobic chains inside its structure, following its binding to the membrane and recognition of the sugar group on the glycolipid. Variation in the residues surrounding the sphingolipid binding site occur between several GLTP homologs [263, 270, 271]. Consequently, electrostatic differences at these sites may be one determinant for the GLTP-membrane interaction, where differently charged lipids may act to regulate the GLTP binding. Tryptophan 142 (W142) has been shown to be essential in the binding of GLTP to the membrane [261, 265, 266, 272–275]. W142 is involved in the initial docking process to the membrane and most likely functions as an interfacial tether that allows for the proper orientation of GLTP in respect to the membrane plane. A third tryptophan residue, W85, is proposed to maintain the proper structural folding of GLTP [273].

The cavity that houses the acyl chains of the bound glycolipid substrate is adaptable and can envelop GSLs of different chain lengths. Analysis of the

crystal structures of GLTPs in either apo- or ligand-bound forms has revealed that the binding of a glycolipid exhibits a structural shift in the alpha-2 and alpha-6 helices, which may relate to the process by which GLTP dissociates from the membrane, following glycolipid detection and uptake [271]. The various chain length GSLs that have been co-crystallized with GLTP exhibit either a "sphingosine-in" conformation, where both acyl chains are embedded within the protein, or a "sphingosine-out" conformation, where the amide-linked chain occupies most of the cavity, subsequently preventing the sphingosine from entering [262]. The sphingosine-out conformation is observable in GLTP structures co-crystallized with longer-chain GSLs, such as 24:1-GalCer. Whether or not these conformations are also present in biological systems remains to be determined, however, a sphingosine-out conformation logically might allow for increased membrane interaction.

The glycolipid specificity, the membrane binding characteristics and the lipid transfer activity of GLTP have been established through various studies, employing several different methods. For example, the utilization of lipid monolayers [276], radiolabel transfer assays [277], fluorescence resonance energy transfer (FRET) assays [278] and recently, surface plasmon resonance (SPR) [275], have resulted in a thorough biochemical characterization of the factors that affect GLTP functionality. Various parameters regarding the membrane that surrounds the glycolipid substrate have been shown to influence the GLTP-mediated transfer, such as membrane lipid composition, curvature and packing. Glycolipid transfer from negatively charged vesicles is significantly slower than from neutral or positively charged vesicles [279]. As GLTP is positively charged at neutral pH (p*I* =9.0), it is plausible that a negatively charged vesicle yields a stronger electrostatic interaction between the protein and the membrane, consequently slowing down GLTP dissociation. GLTP is unable to transfer glycolipids from vesicles made of saturated SMs [280], however, SM analogues that resemble PC allow for transfer to occur. The 3-hydroxy group and the *trans*-4,5 double bond on the sphingosine in SM are determinants for the reduced GLTP-mediated transfer [278]. Membranes consisting of saturated SM allow for transfer, however, at a significantly reduced rate when compared to membranes of chain-matched PC [280, 281].

Membrane curvature seems to significantly affect GLTPs ability to extract glycolipids. Transfer rates are several-fold higher from small, highly curved POPC (1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine) vesicles, when compared to more planar bilayers [282]. Similarly, GLTP does not extract glycolipids from POPC monolayers with biomembrane-like packing [276].

This is perhaps not unexpected, as curved membranes exhibit less membrane packing, allowing for easier glycolipid extraction. Recently, it was shown that GLTP is able to extract glycolipids from high surface-stress planar monolayers that were composed of POPC and physiological amounts of PE or PA [283]. These results somewhat contradict previous studies where negatively charged lipids in POPC vesicles act to inhibit GLTP transfer [279, 282]. However, monolayer systems also allow for lipids to move more freely out of the membrane plane, as there are less hydrophobic forces to keep the lipids in place [271]. Interactions between the glycolipid headgroup and the surrounding membrane presumably also determines the observed effect of adding PE or PA to the membrane, where the smaller PE and PA headgroups allow for increased glycolipid uptake, as the larger headgroup of the PC will act to shield the glycolipids in an "umbrella-like" fashion. Taken together, these results suggest that the biological membrane lipid composition, as well as the curvature of the membrane, may act to regulate GLTP function in cells. Speculatively, GLTP may exhibit a bifunctional role, where it functions as a transporter of lipids at highly curved membrane contact sites (MCSs), and as a sensor at more planar membranes surfaces.

Interestingly, glycolipids are not required to be present in the membranes for GLTP/membrane interaction to occur [275, 282]. It is also not necessary for glycolipids to be embedded in membranes for GLTP/glycolipid-complexation, as it has been observed that GLTP can bind substrates that are dispersed in aqueous solutions, either as monomers or micellar structures [284]. GLTP transfers a variety of glycolipids between model membranes, including GlcCer, GalCer, LacCer and sulfatide, as well as various gangliosides and globosides (reviewed in [10]). SM, cholesterol, PI, PE and PC are not transferred by GLTP in similar systems [277]. A characteristic feature in the glycolipids, that enables their transfer by GLTP, is the β-linkage between the sugar group and the lipid moiety [285]. Early studies suggested that GLTP was able to transfer both sphingoid- and glycerol-based glycolipids, however, the accuracy of these claims has recently been brought into question [10]. Based on the transfer kinetics from the various studies, it would seem that GLTP prefers simpler glycolipids, such as GlcCer, GalCer and LacCer, as its substrates. Ganglioside GM1 seems to be an exception to this rule, and exhibits similar transfer rates to those of the simpler GSLs [10].

2.7.4 Possible *in vivo* functions of GLTP

In the literature, most studies regarding GLTP have focused on its biochemical characteristics, such as its structure, membrane binding

determinants and substrate specificity. The biological role of GLTP is still unclear. GLTP has been shown to be a cytosolic protein [256], which is an important determinant for deciphering its biological function. This is evident when considering that GlcCer is the only GSL that is synthesized on the cytosolic surface of a membrane (see section 2.6.1), making it a prime candidate for GLTP interaction. Additionally, by comparing GLTP to other, biologically more characterized LTPs, one is able to extract subtle hints towards its *in vivo* function.

Several GLTP homologs have been identified, many of which have been implicated in particular cellular functions. The fungal heterokaryon incompatibility protein HET-C2 exhibits high sequence identity to mammalian GLTP [253]. X-ray crystallography analysis of HET-C2 reveals a structure that is highly similar to the mammalian GLTP-fold. HET-C2 has been shown to transfer monohexocslceramides *in vitro*, however at a slower rate than mammalian GLTP [286]. Nevertheless, when comparing to mammalian GLTP, the key amino acid residues in the carbohydrate interacting site are almost completely conserved [253]. In general, genes at the HET *loci* are known to regulate cell-cell interaction events in fungi [287]. As such, a similar function for GLTP might be evident in mammalian cells. In *Arabidopsis thaliana*, two GLTP homologs have been found, namely AtGLTP1 and the accelerated cell death 11 protein (ACD11) [252, 254]. AtGLTP1 is able to transfer GlcCer between model membranes, whereas its ability to translocate GalCer and LacCer is comparatively diminished [252]. AtGLTP1 has been suggested to direct GSLs to the PM, and to possibly control the formation of GSL membrane domains. ACD11 has been shown to accelerate the inter-membrane transport of sphingosine, but not that of GSLs [254]. ACD11 is strongly implicated in apoptosis: when ACD11 is down-regulated, a programmed cell death response is activated [254]. Interestingly, expression of human GLTP in plants with diminished levels of ACD11 leads to a delay in the apoptotic response [288], suggesting a possible role for GLTP in the mammalian apoptotic pathways. Recently, a mammalian GLTP homolog was identified to bind and transfer the phosphorylated ceramide metabolite, C1P [270]. This transfer protein, previously known as human GLTPD1, was named as the ceramide-1-phosphate transfer protein (CPTP). CPTP has structurally a similar fold to that of GLTP, however, CPTP does not transfer GSLs. As such, CPTP seems to hold more in common with the plant ACD11, rather than with mammalian GLTP.

The mammalian four-phosphate adaptor protein 2 (FAPP2) is a protein that contains a GLTP homology domain in its C-terminus. Like GLTP, FAPP2

transfers GlcCer between membranes *in vitro* [183]. FAPP2 has been shown to play an important role in glycolipid events occurring at the *trans*-Golgi network. More specifically, FAPP2 is required for the synthesis of globotriacylceramide (Gb3) [249]. FAPP2 is believed to bind and transfer GlcCer from the cytosolic side of the early Golgi to the later Golgi compartments, where GlcCer is subsequently converted to LacCer on the luminal side of the organelle. Down-regulation of FAPP2 synthesis results in a dramatic decrease in the overall globoside production, whereas ganglioside synthesis remains uninterrupted [249]. As such, FAPP2 plays a key function in the branching pathways of the higher GSLs. Additionally, these findings suggest that at least two different LacCer pools exist in the later Golgi compartments, each of which is destined to function as a precursor pool for a particular type of higher GSL. FAPP2 is also implicated in cellular protein trafficking events, and has been shown to tubulate membranes *in vitro* [289–292].

In contrast to GLTP, which is a single-domain protein, FAPP2 contains additional structural domains. These include the 120 amino acid pleckstrin homology (PH) domain, which is a common structure in many LTPs and other lipid-binding proteins [268, 293]. In FAPP2, the PH-domain specifically recognizes PI4P and the GTPase ADP-ribosylation factor (ARF) on the *trans*-Golgi network [183, 290]. Additionally, PH-domains have been shown to bind to the βγ-subunits of heterotrimeric G proteins, as well as to protein kinase C [294, 295]. Other LTPs that contain a PH-domain include CERT and many of the ORPs. These proteins are likewise implicated in functions involving the Golgi membranes. CERT is responsible for the transfer of ceramide from its site of synthesis at the ER to the Golgi, where ceramide is further modified to produce SM [296]. The founding member of the ORP family, OSBP, is implicated in sterol synthesis as well as in various aspects of sterol regulation [227, 244]. Like FAPP2, both CERT and OSBP are believed to localize to the Golgi through their PH-domains [297, 298].

While GLTP does not contain a PH-domain, it contains a motif that is common to many LTPs. FFAT-motifs (two phenylalanines in an acidic tract) with consensus sequences (EFFDAxE) are known to allow for interaction with the ER-residing VAPs (vesicle-associated membrane protein-associated proteins) [299–301]. Other examples of FFAT-containing LTPs include CERT, FAPP2, OSBP and ORP3 [300, 302–304]. Based on experimental results, variations in some of the amino acids in the consensus FFAT-sequence can be tolerated, regarding VAP interaction [299]. Indeed, GLTP contains two “weak” FFAT-like motifs (**figure 8** and **9**) and has been shown

to bind to VAP-A both *in vitro* and *in vivo* [305] (unpublished results), which would suggest an ER-specific function for GLTP. Of the two motifs, in humans, the upstream one (amino acids 22-28) is weaker than the downstream one (amino acids 32-38), in respect to predicted VAP-binding capacity [304]. It has been suggested that both FFAT-like motifs may be synergistically involved in VAP-binding [304], however, mutation of the downstream motif is enough to inhibit GLTP-VAP-A interaction [305]. In FAPP2, two "weak" FFAT-like motifs are localized similarly to the FFAT-like motifs in GLTP (**figure 9**). Additionally, a third FFAT-like motif exists in mammalian FAPP2 [304]. When the most optimal FFAT-like motif in FAPP2 was tested for ER-interaction in a yeast model system, no targeting could be observed. Phosphorylation of the serine in this FFAT-like motif, however, resulted in weak interaction with VAP-A. Similar phosphorylation of a serine near the FFAT-motif in CERT has been shown to affect its binding to VAP-A [306], suggesting that post-translational modification may be a common method of regulating LTP-VAP interaction.

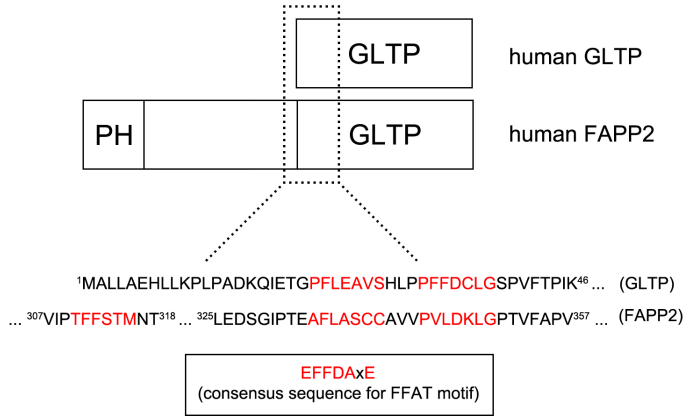

Figure 9. Schematic representation of the human GLTP and FAPP2 secondary structure. The zoom in represents the amino acid sequence from the N-terminal side of the GLTP-domain in each protein. The FFAT-like motifs are shown as red, together with the consensus FFAT motif (boxed). PH = pleckstrin homology domain, GLTP = GLTP domain.

Since GlcCer is the most probable biological substrate for GLTP, it is plausible that GLTP localizes to the cytosolic side of the Golgi compartment, i.e. the site of GlcCer synthesis (**figure 10**). Additionally, it is possible that GLTP interacts with GlcCer that localizes to the ER. In one study, the

FAPP2-dependent synthesis of higher GSLs was suggested to occur through a retroactive transport of GlcCer from the Golgi to the ER [184]. In this model, once GlcCer arrives at the ER, it would be flipped to the luminal side and subsequently be transported back to the Golgi by vesicular means. These findings are supported by a more recent study, where ER-resident flippases were shown to translocate GlcCer between the bilayers of isolated ER membranes [307]. Hypothetically, GLTP might sense GlcCer at the ER and regulate its sorting into vesicles, through its interaction with VAP-A. Indeed, VAP-A has been shown to interact with various SNAREs and, as its name implies, is involved in vesiculation events [308, 309].

Both GLTP and FAPP2 have been implicated in the non-vesicular transport of GSLs to the PM, however, the process by which this occurs is not clear [184]. The non-vesicular transport of GlcCer to the PM was first observed by Warnock et al. some 20 years ago [310]. Despite brefeldin A (BFA) treatment, which results in inhibiton of the secretory transport pathway from the ER to the PM, GlcCer still arrived at the PM in Chinese hamster ovary cells. The non-vesicular trafficking of GlcCer was suggested to be protein-mediated. Later, it was demonstrated that down-regulation of either GLTP or FAPP2 resulted in a decreased transfer of GlcCer to the PM [184]. Neither GLTP- nor FAPP2-down-regulation in BFA-treated cells resulted in a complete inhibition of GlcCer transport to the PM, suggesting that GLTP and FAPP2 may compensate for each other regarding this function. Alternatively, the transport might be taken over by a third, unidentified LTP. Interestingly, a simultaneous knockout of GLTP and FAPP2 did not yield viable cells, suggesting that together, these proteins are essential for cell survival.

The actions of many LTPs may be linked together. Indeed, OSBP has been shown to regulate CERT activity in cells [311]. OSBP is necessary for the formation of a VAP-CERT complex, however, by itself OSBP does not seem to interact strongly with CERT. Consequently, it is not too far fetched to speculate that there may be a wider synergistic function between the various FFAT-motif containing LTPs, where they act together to maintain the lipid homeostasis in cells. FAPP2 and GLTP may very well regulate each other's functions, similarly to that of CERT and OSBP. GLTP and FAPP2 do not seem to exhibit any level of interaction *in vitro*, as assayed by FRET-assays and pull-down experiments (unpublished results). This does not, however, exclude the possibility that GLTP and FAPP2 may interact indirectly, or through mediators, in cells. Other factors, such as post-translational modifications, may also come into play when considering these possible protein-protein interactions.

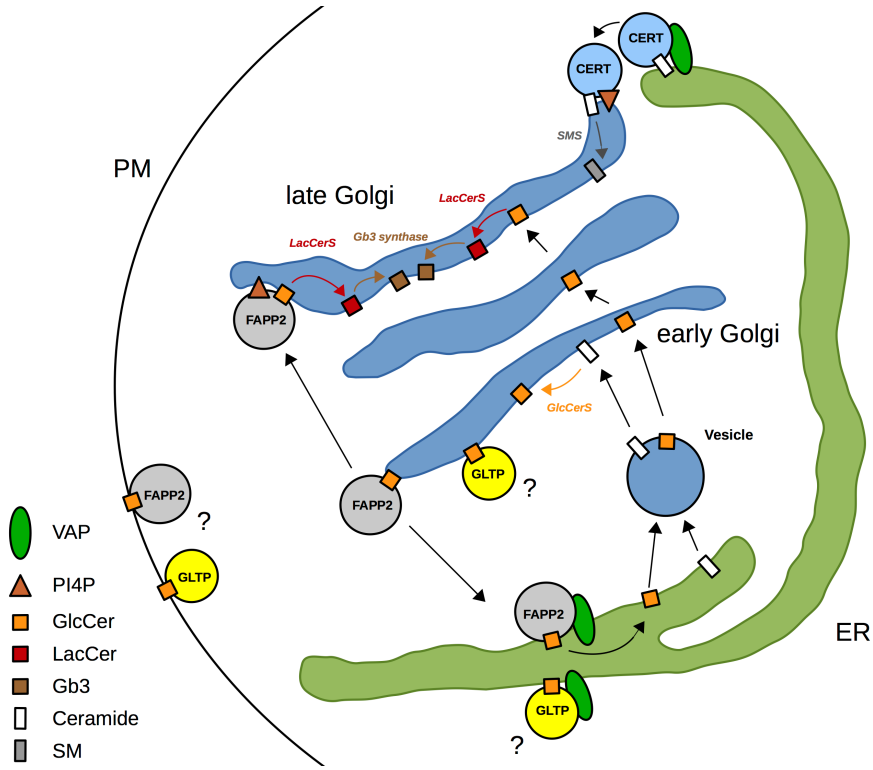

Figure 10. Simplified schematic of established and hypothesized cellular localizations and functions of some sphingolipid transferring proteins. FAPP2 catalyses the transfer of GlcCer from its site of synthesis in the early Golgi to later Golgi compartments for subsequent LacCer and Gb3 synthesis. This occurs either directly, or possibly by a retrograde pathway through the ER. CERT transports ceramide from the ER to the *trans*-Golgi, presumably at ER-Golgi contact sites. FAPP2 and CERT associate with PI4P on the Golgi membrane through their PI homology domain. Both GLTP and FAPP2 have been implicated in the transfer of GSLs to the PM. GLTP may bind GlcCer at the Golgi or possibly at the ER. CERT interacts with VAPs at the ER. Both GLTP and FAPP2 might also exhibit VAP interaction.

3. AIMS OF THE STUDY

The general aims of the work done for this thesis were twofold. Firstly, the role of GLTP was to be examined in relation to GSL metabolism in cells. Secondly, a new ceramide delivery system for cells in culture was to be studied and developed. More specific aims of each of the publications are listed below.

The main aim of **publication I** was to alter the cellular GSL content, and to subsequently study what effects these alterations have on the cell, in respect to GLTP expression and protein levels. Inhibitors of vesicular transport and sphingolipid synthesis, as well as RNA interference, were utilized for this purpose. In **publication II**, the aim was to analyse the lipidome of cells with altered GLTP expression. Cellular GLTP levels were genetically up- or down-regulated and the changes in the cellular lipidome were examined.

The aim of **publication III** was to study and develop a solvent-free method for introducing various chain length ceramides to cultured cells. The ceramides were complexed with cholesteryl phosphocholine to form stable bilayer vesicles, and were subsequently introduced to cultured cells by addition to the growth medium. The ceramide uptake, the effects that the ceramides had on cell proliferation, as well as the cellular fate of the introduced ceramides, were analysed.

In addition, unpublished results are discussed and presented in this thesis, regarding data that supports the various observations made in the published works.

4. EXPERIMENTAL PROCEDURES

A brief summary of the experimental procedures is presented here. A more comprehensive overview of the different methods used in this thesis can be found in each of the original publications.

4.1 Materials and chemicals (I, II and III)

All chemical reagents were of analytical grade or higher. Lipid standards where from Avanti Polar Lipids (Alabaster, USA) or Matreya LLC (Pleasant Gap, USA). Cholesteryl phosphocholine was synthesized as described by Lönnfors et al. [312], or obtained from Avanti Polar Lipids. D-erythro-sphingosine and hexanoic acid were purchased from Larodan (Malmö, Sweden). [3-^{3}H]D-erythro-sphingosine and [9-10,^{3}H]hexadecanoic acid were obtained from PerkinElmer (Waltham, MA, USA). Decanoic and hexadecanoic acids were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and organic solvents were from Rathburn Chemicals Ltd (Walkerburn, Scotland) or Avantor Performance Materials (Center Valley, PA USA). Monensin, brefeldin A and N-butyldeoxynojirimycin were obtained from Toronto Research Chemicals (North York, ON, Canada). Myriocin, 1-phenyl–2-decanoylamino-3-morpholino-1-propanol and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA). [4, 5-^{3}H]-sphinganine was a kind gift from Dr. Tony Futerman (Weizmann Institute of Science, Israel). Conduritol-B-epoxide was purchased from Merck Chemicals (Calbiochem) (Darmstadt, Germany) and tunicamycin was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Chloroquine and ceranib-2 were purchased from Sigma-Aldrich and fumonisin B1 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The polyclonal rabbit antibody against GLTP has previously been described [256]. The rabbit anti-beta actin antibody was from Rockland Immunochemicals (Gilbertsville, PA, USA). The rabbit anti-GlcCer and anti-GalCer antibodies were from Glycobiotech, GmbH (Germany). The secondary peroxidase-conjugated anti-rabbit antibody was from Thermo Scientific (Waltham, MA, USA). The expression plasmid encoding human GLTP has been described previously [256]. The empty green fluorescent protein vector (pEGFPN1) was from Clontech (Palo Alto, CA, USA). The various siRNAs, the universal negative control siRNA and the fluorescein isothiocyanate labeled (FITC) dsRNA oligomers were from Invitrogen (Carlsbad, CA, USA). The primer pairs used for qPCR analysis were obtained from DNA Technology A/S (Risskov, Denmark). The Universal ProbeLibrary probes, likewise used in the qPCR experiments, were obtained from Roche Diagnostics (Basel, Switzerland).

4.2 Cell Culture (I, II and III)

Human skin fibroblasts (GM08333) were obtained from Coriell Institute for Medical Research (Camden, NJ, USA). HeLa cells (ATCC CCL-2, LGC Standards) were a kind gift from Dr. Lea Sistonen (Åbo Akademi University, Finland). All cells were grown (and treated, unless otherwise stated) in Dulbecco's modified Eagle's medium, supplemented with penicillin/streptomycin, 2 mM L-glutamine and 10% fetal calf serum (Sigma-Aldrich), at 37°C in a humid environment with 5% CO_2.

4.3 Transient transfection of plasmids and siRNA (I and II)

All plasmid transfections were performed using a BIO-RAD Gene Pulser II RF Module electroporator. Lipofectamine 2000 (Invitrogen) was used to transfect cells with siRNA, according to the manufacturers instructions.

4.4 RNA extraction, reverse transcription and quantitative real time PCR analysis (I and II)

Total RNA was isolated from cells using a NucleoSpin RNA II (Macherey-Nagel, Germany) RNA-extraction kit, according to the manufacturer's instruction. cDNA was obtained through reverse transcription of the purified RNA, which was carried out using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The cDNA was amplified and quantified by performing quantitative real time PCR (qPCR). qPCR was performed by the staff at the Turku Centre for Biotechnology using the Applied Biosystems 7900 HT Fast Sequence Detection System.

4.5 Flow Cytometry and Cell Sorting (II)

The FITC (siRNA) and GFP (GLTP overexpression) positive cells were sorted from the untransfected cells to ensure a reliable lipidomics analysis. The flow cytometry was performed at the Cell Imaging Core facility at the Turku Centre for Biotechnology, Turku, Finland.

4.6 Mass spectrometry analysis (II)

The lipid extraction of the cell samples and the lipidomics analyses were conducted by Zora Biosciences Oy (Espoo, Finland) according to their standard operating procedures. The species of all phospholipids, SM, DAG and CE were analysed by shotgun analysis on a hybrid triple

quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB SCIEX, MA) equipped with a robotic nanoflow ion source (NanoMate HD, Advion Biosciences, NY). Sphingolipids were analysed by reverse phase ultra-high pressure liquid chromatography (UHPLC) using an Acquity BEH C18, 2.1×50 mm column with a particle size of 1.7 µm (Waters, Milford, MA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB SCIEX, MA). Lipidomic data is based on the analysis of each detected lipid class with one technical replicate for each cell sample. The samples, the CTRL cells, GLTP siRNA and GLTP OE cells were in duplicates.

4.7 Western blot analysis (I and II)

Cells were collected by trypsination, and washed twice in PBS. The pelleted cells were redissolved in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween-20, 0.5 mM PMSF, 1×Protease inhibitor cocktail (Sigma), 1 mM dithiotreithol, pH 8.0). The cells were sonicated using a Branson 250 probe sonifier (Emerson Industrial Automation, St. Louis, MO, USA), and the lysate protein concentration was determined using the method of Lowry [313]. Appropriate amounts of the cell lysates were separated on SDS–PAGE and transferred onto a PVDF membrane. Immunoblots were labeled using antibodies against GLTP and β-actin. The detected proteins were visualized with the ECL chemiluminescence system (SuperSignal West Femto, Thermo Scientific) using X-ray film (Fujifilm, Tokyo, Japan).

4.8 Synthesis of ^{3}H-labeled ceramides (III)

^{3}H-C6-, ^{3}H*C10-, and ^{3}H-C16-ceramide were prepared from [3-^{3}H]D-erythro-sphingosine and hexanoic, decanoic and hexadecanoic acids, respectively, using *N,N′*-dicyclohexylcarbodiimide and triethylamine as catalysts [314]. [^{3}H]palmitoyl ceramide was prepared from sphingosine and [9-10,^{3}H]hexadecanoic acid, using *N,N′*-dicyclohexylcarbodiimide and tri-ethylamine as catalysts [42]. The products were purified by preparative HPLC on a C18 phase, using methanol as solvent. Purity was assessed by analytical HPLC, and molecular identity by ESI-MS.

4.9 Preparation of ceramide-cholesterol phosphocholine bilayers (III)

CholPC and ceramide stocks were kept in hexane-isopropanol (3:2 by volume) solutions and stored at -20°C until used. Ceramide/CholPC

complexes of desired concentration were prepared from the stock solutions. The appropriate amount of each lipid was dried under nitrogen in a glass tube, redissolved in chloroform to ensure proper lipid mixing and dried again. The dehydrated lipid film was hydrated in PBS, pH 7.4, at 55°C for 20 minutes and then sonicated for 5 minutes in a FinnSonic M3 bath sonicator (FinnSonic Oy, Lahti, Finland) at the same temperature. The solution was further sonicated for 10 minutes using a Branson 250 probe sonifier at room temperature (Emerson Industrial Automation, St. Louis, MO, USA). The resulting clear solution was then immediately centrifuged at 12 000 rpm for 10 minutes using a tabletop microfuge, to remove titanium probe particles and any undispersed lipids. The solution was transferred to a glass tube and kept at RT and used within 1 h. Prior to use, the ceramide concentration in the solution was re-calculated based on the specific activity of the radiolabeled ceramide.

4.10 Metabolic labeling and HPTLC analysis of cellular lipids (I, II and III)

Cells were treated with radioactive lipids as describes in the publications (I – III). Following treatment, cell dishes were washed twice with PBS and dried to completion in a fume hood. Total lipids were extracted directly from the cell dishes using hexane:isopropanol (3:2 by volume). Lipid extracts were dried under a stream of nitrogen and redissolved in appropriate volumes of hexane:isopropanol. Appropriate volumes of each sample were applied on high performance thin-layer chromatography (HPTLC) silica plates (Whatman, UK). For GlcCer, GalCer and LacCer separation the solvent system chloroform:methanol:acetone:acetic acid:water (10:2:4:2:1) was used. For Gb3 separation the solvent system 45:55:10, chloroform:methanol:0.2% $CaCl_2$ (in H_2O) was used. For phospholipids, chloroform:methanol:acetic acid:water; 50:30:8:3 was used as the as the solvent system. The analysis was done using standards, run in parallel with the samples. GSL migration was visualized using orcinol spray (0.2% orcinol in a 50% H_2SO4 solution) and heating the plate on 120°C for 5 minutes. Alternatively, iodine staining or cupric acid staining was used. The lipid spots were scraped into Optiphase 'Hi phase' scintillation liquid (PerkinElmer-Wallac, Turku, Finland) and the radioactivity was measured using a liquid scintillation counter, 1216 Rackbeta (PerkinElmer-Wallac, Turku, Finland). After lipid extraction, the total cellular proteins were extracted with 0.1 M NaOH and the protein content was analysed with the Lowry method [313]. The counts per minute (cpm) obtained were either normalized to the total protein content for each respective sample, or presented as a percentage of the total signal per sample (total cpm in sample lane).

4.11 Dot blot analysis of cellular GlcCer and GalCer (III)

High-dose C6- and C10-ceramide (50 μM and 100 μM, respectively) treated samples were analysed by HPTLC as described above. The HPTLC plate was stained by iodine vapour, and the lipid spots corresponding to C6- and C10-GlcCer were scraped into glass tubes. The lipids were extracted from the scraped silica using chloroform-methanol (2:1 by volume) and dried under a nitrogen stream. The dried lipids were redissolved in hexane-isopropanol (3:2 by volume) and appropriate amounts were dotted onto a nitrocellulose membrane, alongside GlcCer and GalCer standards (2 nmol/dot). The lipids were visualized by immunoblotting, using commercial rabbit anti-GlcCer and rabbit anti-GalCer antibodies (Glycobiotech GmbH), an HRP-conjugated anti-rabbit secondary antibody (Thermo Scientific) and enhanced chemiluminescence detection (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific).

4.12 Cell viability assay (III)

A resazurin reduction assay (alarmaBlue, Life Technologies) was used to investigate cytotoxicity of C6-, C10-, and C16-ceramide. The cells were treated for 22 hours with the different ceramides, cholesterol or a PBS-vehicle. After the 22-hour incubation, the resazurin conversion assay was performed according to the manufacturer's instruction. The fluorescence was measured by a Varioskan Flash Multimode Reader (Thermo Scientific) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

5. RESULTS AND DISCUSSION

5.1 GLTP expression correlates with cellular GSL synthesis (I and II)

While the structural and membrane-binding properties of GLTP, as well as many other of its *in vitro* capabilities have been studied quite extensively [10, 226, 260, 261, 266, 271, 315], the biological function of GLTP remains elusive. Previous studies have revealed that GLTP, like many other LTPs, is localized to the cytosol [256]. In addition to localizing to the cytosol, GLTP has been shown to interact with the integral ER membrane protein VAP-A [305]. The cellular localization of GLTP is of great importance when considering its possible protein interaction partners, as well as which lipids would function as its most probable ligands. It has become widely accepted that most of the GSLs are synthesized on the luminal side of the Golgi apparatus, with the exception of GlcCer, which is produced on the cytosolic side [177–182]. Considering its cytosolic nature, the most probable lipid substrate for GLTP *in vivo* is GlcCer, since GLTP would be readily available to interact with the lipid at the cytosolic side of the Golgi, immediately after its synthesis. Alternatively, GLTP might be able to interact with GlcCer at the cytosolic side of the ER (or perhaps the ER/Golgi intermediate compartment), following FAPP2-mediated GlcCer translocation [184]. Based on the above-mentioned findings, we have hypothesized that GLTP might be involved in maintaining the cellular glycolipid homeostasis, where GlcCer would be its most probable interaction candidate. In an effort to shed light on GLTPs biological function, we set out to examine the GLTP-glycolipid connection in more detail.

5.1.1 Inhibitors of vesicular transport increase GLTP expression (I)

In the early stages of my thesis work, I observed that treatment of cells with the fungal macrolide brefeldin A (BFA) resulted in a several-fold increase in GLTP expression. Warnock et al. had previously reported that, while BFA treatment effectively inhibited the vesicular transport of lipids and proteins to the PM, the transport of GlcCer was retained [310]. The authors postulated that, in the absence of vesicular trafficking, GlcCer might be transported by non-vesicular means, perhaps with the help of LTPs. Halter and coworkers later expanded on the idea and demonstrated the possibility of two glycolipid transporting proteins (namely GLTP and FAPP2) being involved in this trafficking [184]. These studies promted us to take a closer look at how GLTP expression is affected in cells that are treated with BFA and other vesicular transport inhibitors, as well as inhibitors of GSL synthesis and degradation.

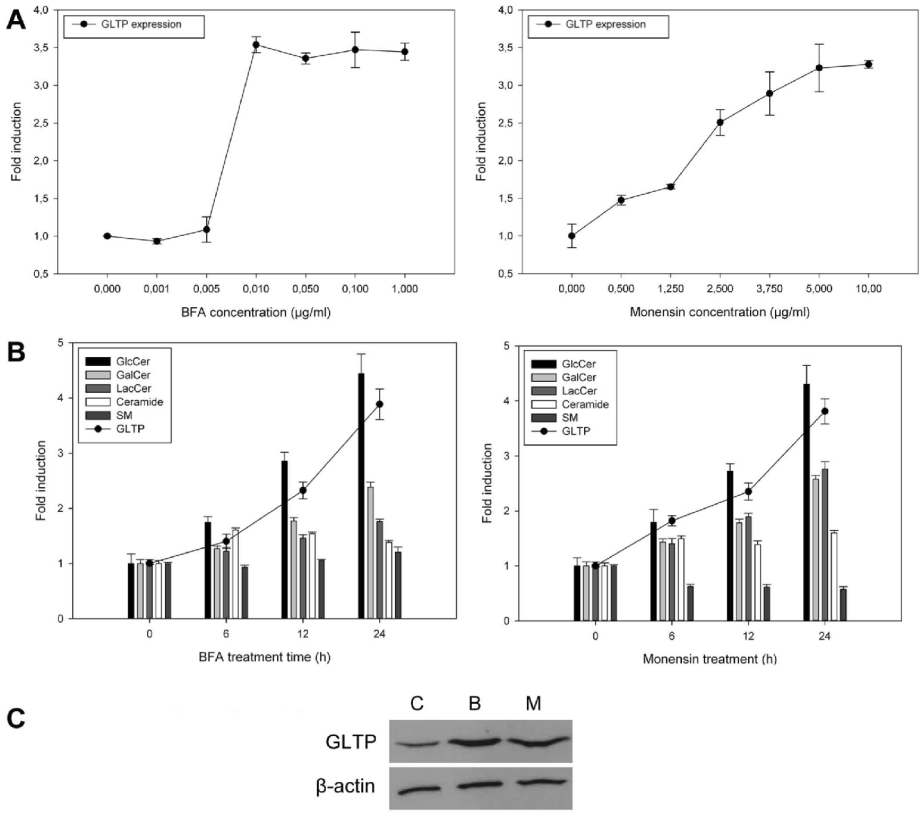

Figure 11. Effects of BFA and monensin treatment on GLTP expression, GLTP protein levels and ^{3}H-sphinganine incorporation into GlcCer, GalCer, LacCer, ceramide and SM in HSF cells. **A**) qPCR analysis of GLTP expression in cells treated with varying concentrations of BFA (**left panel**) or monensin (**right panel**) for 24 hours. **B**) qPCR analysis of GLTP expression (filled circles) and HPTLC analysis of radiolabeled sphingolipids in cells treated with BFA (0.01 µg/ml, left panel) or monensin (5 µg/ml, right panel) for 6, 12 and 24 hours. **C**) Western blot analysis of GLTP levels in cells treated with BFA (0.01 µg/ml) or monensin (5 µg/ml) for 24 hours. C=untreated control, B=BFA treatment, M=monensin treatment. Beta-actin was used as a loading control. Radiolabeling and qPCR results are means +/- SD of at least 3 independent experiments. Figure is adapted from publication I.

In our studies, we found that a 24-hour treatment of HSF cells with two vesicular transport inhibitors (BFA and monensin) resulted in increased GLTP mRNA levels (**figure 11A**), as analysed by qPCR. In the case of monensin treatment, the increase in expression was concentration dependent, whereas for BFA treatment, the expression peaked rapidly at a low concentration. HPTLC and qPCR analysis of cells treated with BFA and monensin demonstated a time-dependent increase of GLTP expression and ^{3}H-sphinganine incorporation into GSLs (**figure 11B**). The radiolabeling results were well in accordance with previous findings [316–319]. Similarly, BFA and monensin treatment also increased the total mass of GSLs in cells (I – figure 2). The inhibitors also increased the total GLTP levels in cells, as assayed by Western blotting (**figure 11C**).

The two different inhibitors act in different ways regarding how they increase radiolabel incorporation into GSLs. BFA inhibits vesicular transfer of proteins and lipids to the PM by disrupting vesicle coat assembly and by inducing retrograde protein transport from the Golgi apparatus to the ER. This subsequently results in the fusion of the two organelles, creating an ER-Golgi complex, while leaving the *trans*-Golgi network fused with the late endosomes [320, 321]. The inhibition of vesicular transport presumably leads to an accumulation of GSLs in the fused ER-Golgi complex. Monensin is a monovalent ionophore that is known to interfere with vesicular transport through the Golgi apparatus [319, 322]. Monensin has been shown to inhibit the synthesis of SM while increasing radiolabel incorporation into ceramide and the GSLs. Our data indicates that the increased synthesis of the various GSLs, that results from treatment with these compounds, correlates well with the GLTP expression (**figure 11B**). It is possible that the increased GSL synthesis, particularly that of GlcCer, directly results in the cells compensating for the increased GSL levels by up-regulating GLTP expression. This would support previous suggestions regarding GLTPs putative role as a GSL reporter, sensor and/or transporter.

5.1.2 Lysosomal accumulation of GlcCer does not affect GLTP expression (I)

When cells were treated with BFA and monensin, GlcCer demonstrated the highest accumulation of the analysed GSLs, both according to absolute radiolabeling values (data not shown) as well as based on HPTLC lipid mass analysis (I – figure 2). In this study, we did not analyse the radiolabeling or mass changes of some of the more complex GSLs (e.g. the globo- and ganglio-classes). However, the literature describes the effects that BFA and monensin have on higher GSL metabolism fairly comprehensively [168]. In

general, the inhibitors severely impede the membrane flow from the proximal to the distal Golgi compartments, leading to an immobilization and subsequent accumulation of less complex GSLs (such as GlcCer, LacCer and GM3), whereas the synthesis of the more complex GSLs is inhibited through topological limitation of available precursor lipids [168].

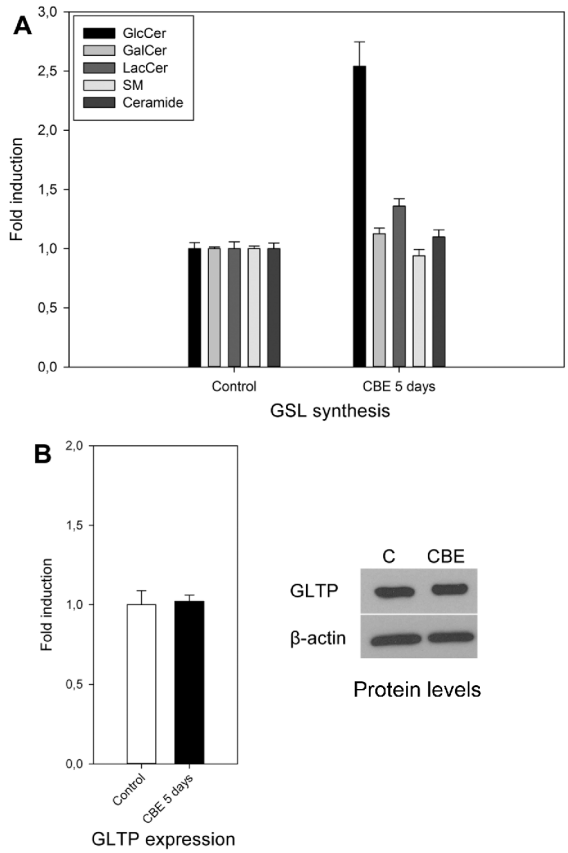

Figure 12. Effect of CBE treatment on GLTP mRNA and protein levels, as well as on radiolabel incorporation into sphingolipids. HSF cells were treated with CBE (250 µM) for 5 days. **A**) Simple sphingolipid levels were determined by ^{3}H-sphinganine incorporation. **B**) GLTP expression (**left**) and protein levels (**right**) as determined by qPCR and Western blotting, respectively. Radiolabeling and qPCR results are means +/- SD of at least 3 independent experiments. Figure is adapted from publication I.

The accumulation of GlcCer in the ER, Golgi and ER/Golgi complex in these experiments, could explain the observed increase in GLTP expression. In an effort to elucidate whether GLTP expression is affected by *de novo* synthesis of GlcCer in particular, rather than an overall accumulation of this lipid (for example as a result of inhibited lysosomal degradation) we used conduritol-beta-epoxide (CBE). CBE is an inhibitor of beta-glucosidase, an enzyme primarily responsible for degradation of GlcCer in the lysosomes [323]. A 5-day CBE treatment led to a significant increase in radiolabel incorporation into GlcCer, as well as an increase of GlcCer mass (**figure 12A** and I – figure 4C). However, neither GLTP mRNA nor protein levels were affected by the treatment (**figure 12B**). This suggests that GLTP is more likely to be involved in events where GlcCer levels are elevated due to increased synthesis, rather than due to inhibited degradation.

5.1.3 Inhibition of GlcCer synthesis decreases GLTP expression (I)

In an effort to further verify that the observed effects were indeed due to an increase in *de novo* GSL synthesis, we utilized two GlcCer synthesis inhibitors (n-butyldeoxy-nojirimycin [NB-DNJ] and 1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol [PDMP]) [324, 325], as well as an inhibitor of serine palmitoyl transferase (myriocin) [326]. The assumption was that if the BFA- and monensin-mediated increases in *de novo* GSL synthesis were to be impeded by simultaneous exposure to GSL synthesis inhibitors, a subsequent decrease in the GLTP expression should also be observable, when comparing to the BFA and monensin treatments alone. **Figure 13** shows that, with the exception of the BFA+NB-DNJ treated cells, all co-treatment experiments demonstrated a clear decrease in GLTP expression that seemed to correlate well with the inhibited GlcCer radiolabeling. Interestingly, and perhaps even paradoxically, both NB-DNJ and PDMP treatment also decreased radiolabel incorporation into GalCer. The observation that GalCer radiolabeling was not inhibited in the BFA+myriocin treated cells was also mystifying.

Treatment with GSL synthesis inhibitors alone also led to decreases GLTP expression (I – figure 6). Here, again, treatment with NB-DNJ and PDMP decreased GalCer radiolabeling. Interestingly, longer substrate exposure times (72 h), using myriocin, were required to observe changes in GLTP protein levels. The semi-quantifiable nature of traditional Western blotting may not tell the whole story, as minor changes in protein levels are difficult to detect on blots. NB-DNJ and PDMP treatment alone also resulted in similarly decreased GLTP levels, after 72 hours of treatment (unpublished results).

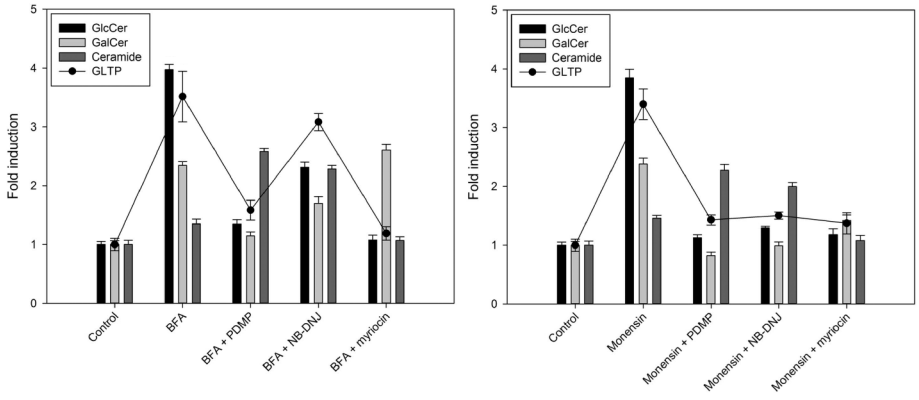

Figure 13. Left panel: Sphingolipid radioactivity (bars) and GLTP mRNA levels (filled circles) in HSF cells treated with either BFA alone (0.01 µg/ml), or co-treated with BFA and PDMP (50 µM), NB-DNJ (250 µM) or myriocin (25 µM). **Right panel:** Same as left panel, but instead of BFA, monensin was used. ^{3}H-sphinganine was used for radiolabeling, with the exception of the myriocin experiments, where ^{3}H-palmitic acid was used. Radiolabeling and qPCR results are means +/- SD of at least 3 independent experiments. Figure is adapted from publication I.

Substrates that affect major cellular processes can cause a myriad of unpredictable effects on cells. This is a significant methodological limitation when attempting to analyse isolated cellular functions, especially in the case of BFA and monensin treatment, where the cells are subjected to structural changes in the ER and Golgi compartments. While ER stress-induction experiments did not seem to alter GLTP expression significantly (I – figure 10), some of the more unexpected results obtained in this study may be explained by possible non-specific effects that the different inhibitors may have had on the cells, especially when considering the co-administration experiments. Increased specificity regarding alteration of cellular processes may be acquired by the use of more direct approaches, such as gene regulation by RNA interference. To study the possible GSL-GLTP connection more specifically, short-interfering RNA (siRNA) designed to down-regulate GlcCerS synthesis was transfected into cells. The expression of the GlcCerS gene was down-regulated by approximately 80%, based on qPCR analysis. Subsequently, an 80% decrease in GlcCer radiolabeling was also observed, as well as a less drastic decrease in GalCer and LacCer radiolabeling (I – figure 7). Knockdown of GlcCerS also had an effect on GLTP mRNA and protein levels, where a clear reduction of both could be observed (I – figure 8). The method of GlcCerS down-regulation was further employed in combination

with BFA and monensin treatment, where the knockdown of GlcCerS expression resulted in decreased GSL synthesis and GLTP expression, even in the presence of BFA and monensin (**figure 14**).

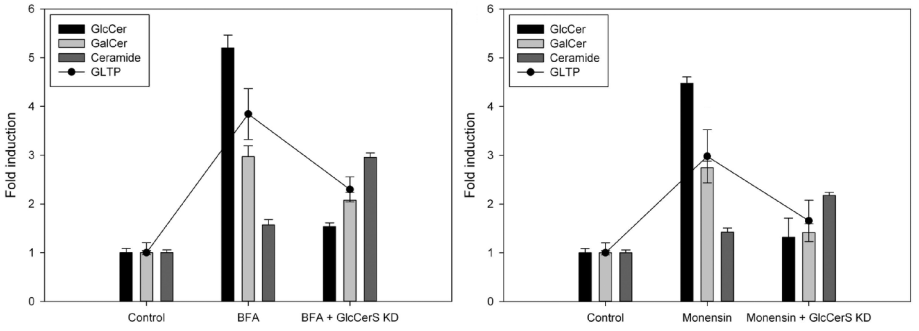

Figure 14. ^{3}H-sphinganine incorporation into GlcCer, GalCer and ceramide (bars) as well as GLTP mRNA levels (filled circles) in GlcCerS down-regulated HSF cells, treated with BFA (**left**, 0.01 µg/ml) or with monensin (**right**, 5 µg/ml). Radiolabeling and qPCR results are means +/- SD of at least 3 independent experiments. Figure is adapted from publication I.

The observation that both the GlcCerS-specific inhibitors PDMP and NB-DNJ, as well as the specific down-regulation of GlcCerS expression by RNAi, markedly reduced GalCer synthesis was unexpected. This raised some concern regarding the methodology used in the publication. At first, it was assumed that the unexpected results may simply have been due to unpredictable secondary reactions that the various inhibitors may have had, alone, or particularly, when co-administered. There also exists some discrepancy regarding how well different GlcCerS inhibitors actually reduce the synthesis of various GSLs [327]. Additionally, while RNA interference may be seen as a more direct approach in regulating GlcCer levels, it is not without its own problems. GlcCerS is a key enzyme in an important metabolic pathway, and its knockdown may have several unpredictable effects on the cell. Off-target down-regulation of other genes may also be involved. Most important, however, was the possibility that the HPTLC-based method was not efficient in separating the endogenous GlcCer and GalCer lipid species from each other, and that as a result, contamination between the lipid spots could have occurred. Immuno-analysis of the separated spots using highly specific anti-GlcCer and anti-GalCer antibodies, however, revealed that only minor cross-contamination in the separated lipids spots was

detectable (data not shown). In addition, based on the immuno-analysis, it became apparent that GlcCer clearly is the major monohexosylceramide species in HSF cells. The results indicated that 80 – 90% of the monohexosylceramides consisted of GlcCer. Borate-impregnation of HPTLC-plates, which increases the resolution of GlcCer separation from GalCer [328], did not yield differing results from the ones presented in this thesis (data not shown). Still, care should be practiced when interpreting the results, especially regarding how the GlcCer and GalCer levels correlate to each other in the various experiments.

5.1.4 Summarizing notes (I)

Taken together, the results presented in publication I show that there is an apparent connection between GSL and GLTP levels in cells. The cells seem to up-regulate GLTP expression when there is an increased *de novo* synthesis and/or accumulation of GlcCer at or near its site of synthesis. In contrast, when the production of GlcCer is inhibited, GLTP expression is decreased. The results support previous suggestions regarding GLTP's possible function as a sensor or reporter of GlcCer levels in cells [256, 284]. GlcCerSs have been found in a variety of organisms, ranging from animals to plants and fungi [164, 165]. Interestingly, some organisms that lack GlcCerS, for example S*accharomyces cerevisiae* and *Schizosaccharomyces pombe*, also lack GLTP-like proteins [252]. Although this rule is not applicable in all cases, there appears to be a fundamental genetic link and co-occurrence between GlcCerS and GLTP.

It is tempting to speculate that GLTP may be involved in the transport of GlcCer in cells, either directly or indirectly through interaction with other lipid transport mechanisms. With the help of its ER-targeting FFAT-like motif, GLTP might be able to direct GlcCer away from the ER to other cellular destinations, such as the PM, or to the Golgi for further glycosylation. As mentioned before, BFA treatment blocks vesicular transport to the PM, inhibiting the trafficking of lipids and other cargo. GlcCer, however, seems to retain its transport to the PM, despite the absence of a functioning vesicular transport system. This non-vesicular transport of GlcCer to the PM has been postulated to be protein-mediated [310], and may in part be carried out by GLTP [184]. It would seem logical that inhibition of the vesicular transport system requires an up-regulation of alternative transport methods for maintaining GlcCer transport to the PM, which in turn could explain the up-regulation of GLTP expression under these conditions. It is important to note, however, that the results presented here do not eliminate the possibility that

the observed effects, regarding GLTP mRNA and protein levels, are due to changes in other GSLs, such as GalCer or LacCer.

5.1.5 Altering GLTP expression causes changes in the cellular lipidome (II)

In publication I, we examined how alteration of GSL metabolism affects GLTP expression and protein levels. In publication II, we instead analysed how up- or down-regulation of GLTP levels affect the cellular lipidome. Previously, it has been reported that transient up-regulation of GLTP expression in HeLa cells led to an increase in radiolabeled GlcCer, whereas a transient knockdown of the *gltp* gene did not result in significant changes in GlcCer radiolabeling [256]. In publication II, we expanded upon these findings by studying a broader range of lipids, using metabolic labeling and HPTLC analysis, complemented by a lipidomics MS approach. Overall, 15 different lipid classes, including a total of 142 lipid species, were quantified. The MS analysis revealed changes in eight lipid species, which presumably resulted from either up- or down-regulation of GLTP. These lipids included GlcCer, LacCer, Gb3, ceramide, SM, cholesterol-esters (CE), diacylglycerol (DAG) and PS.

The two methods of lipid analysis used in publication II differ from each other significantly, and these differences should be kept in mind when considering the results. The HPTLC data represents all of the chain-specific lipids of each particular lipid type analysed, which migrate in close proximity to each other on the HPTLC plate (at least when using the solvent systems described in section 4.10), and are pooled for subsequent analysis. MS methodologies allow for analysis of the various chain-specific lipid species separately. When considering the HPTLC data in this study, it should also be kept in mind that radiolabel incorporation into lipids does not necessarily correspond directly with lipid mass. As such, direct parallels between the MS and the HPTLC data should be drawn with caution. Additionally, it should be noted that in the MS analysis, GlcCer and GalCer were not distinguishable from each other, using the methods described in section 4.6. The HPTLC methods were able to separate these lipids for analysis.

5.1.6 GSL levels are affected by changes in GLTP expression (II)

Our results are in accordance with previous findings, where GLTP up-regulation led to an increase in GlcCer ^{3}H-sphinganine radiolabeling and knockdown of GLTP resulted in no significant changes in GlcCer levels (**figure 15** and **16A**) [256]. Differing from previous works, however, were the

results that showed an increase in LacCer levels in cells overexpressing GLTP, as well as a decrease in cells with down-regulated GLTP synthesis. This was apparent from both the MS and HPTLC analysis, however, in the radiolabeling experiments the changes were not as pronounced (**figure 15** and **16A**). A novel finding was that both up- and down-regulation of GLTP caused significant, positive correlation in the cellular Gb3 levels (**figure 15** and **16B**). D'Angelo and co-workers have previously demonstrated similar effects when silencing FAPP2 expression [183, 249]. In their work, FAPP2 depletion resulted in decreased LacCer and Gb3 synthesis, while GM3 levels remained unchanged. The authors suggest that FAPP2 mediates the transfer of GlcCer to distal Golgi compartments, specifically for the subsequent synthesis of LacCer and Gb3, but not GM3. These results indicate that at least two different LacCer pools exist in the Golgi apparatus, one that is used for ganglioside synthesis and another that is used for globoside synthesis. Previously, Lingwood et al. have also suggested that neutral and acid GSLs are generated from two distinct precursor pools [187]. GlcCer destined for LacCer synthesis in the early Golgi is presumably transported by vesicular means [183].

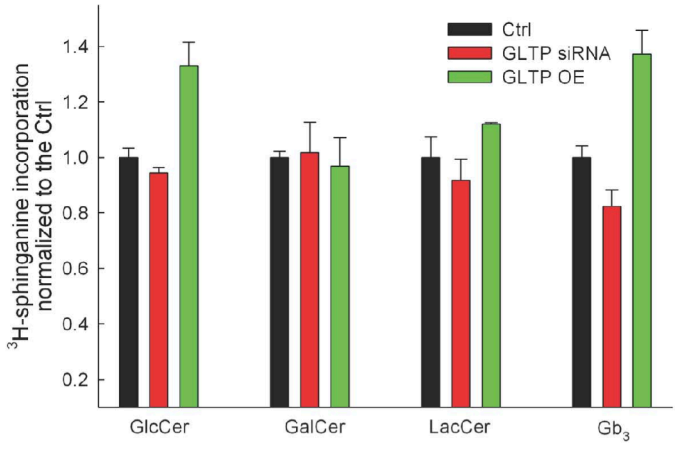

Figure 15. HPTLC analysis of HeLa cells with down- or up-regulated GLTP expression. Metabolic labeling of HeLa cells with ^{3}H-sphinganine, control (black), GLTP siRNA (red) and overexpression of GLTP (green). Radiolabeling results are means +/- SD of at least 3 independent experiments. Figure is adapted from publication II.

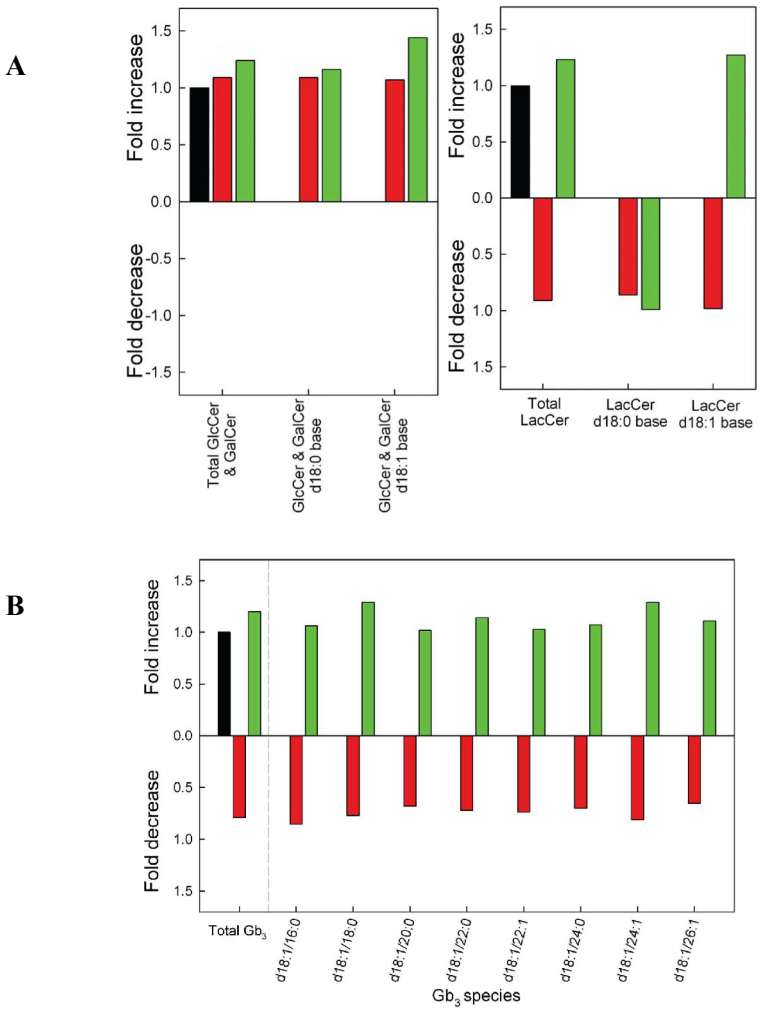

Figure 16. Relative mass changes in lipid species of **A)** GlcCer/Galcer, LacCer and **B)** Gb3 after knockdown (red) or overexpression (green) of GLTP in HeLa cells, as analysed by MS. Black = control. Figure is adapted from publication II.

The increase in Gb3 levels that occurs as a result of up-regulating GLTP expression could be a direct consequence of the overall increase in GlcCer synthesis that also takes place. Controversially, however, down-regulation of GLTP in cells does not affect GlcCer levels significantly, whereas Gb3

synthesis is still markedly reduced. Previously, FAPP2-ablation has been shown to increase GlcCer levels in cells [183, 184]. This is presumably due to an accumulation of GlcCer in the earlier compartments along the GSL synthesizing membranes, as FAPP2-mediated transport of GlcCer to distal Golgi compartments does not occur.

Taken together, the results suggest that FAPP2 and GLTP affect Gb3 synthesis by different means. Considering that GLTP knockdown does not affect GlcCer synthesis significantly, it is possible that GLTP does not function as a direct transporter of GlcCer in cells, but rather as a sensor or reporter of GlcCer levels. The apparent involvement of both proteins in Gb3-metabolism also raises the possibility for cellular interaction. Indeed, increased levels of GLTP at the ER/early Golgi could lead to an increase in the directing or sensing of GlcCer for FAPP2-mediated transport, resulting in more GlcCer arriving at the late Golgi compartment for subsequent LacCer and Gb3 production. Decreased levels of GLTP would, on the other hand, have the opposite effect on Gb3 synthesis. As gangliosides were not analysed in this work, their relation to GLTP expression remains unknown.

5.1.7 Alterations in GLTP expression affect the levels of non-GSLs (II)

According to the MS data, the overall ceramide levels were only marginally affected by up- or down-regulation of GLTP. Cells overexpressing GLTP showed, however, a clear reduction in the d18:0-base ceramides (II – figure 3E). The MS data also shows that total SM levels were decreased by both GLTP overexpression and knowckdown (II – figure 5A). Here, again, a clear reduction of d18:0-base SM was observable in the GLTP down-regulated samples. Radiolabeling of SM only showed a slight decrease in comparable experiments (II – figure 2). SM synthesis is mediated by at least two different SM synthases, SMS1 and SMS2 [329]. The SMSs catalyse the exchange of phosphocholine from PC to the hydroxyl group of ceramide, yielding SM and DAG. SM synthesis is largely dependent of the ceramide transporter CERT, which transports ceramide from the ER to the Golgi apparatus [247, 330]. Interestingly, CERT is also capable of binding DAG, albeit to a lesser degree when compared to ceramide [247, 331]. Still, it has been proposed that CERT might transport DAG from the Golgi back to the ER [331]. The MS data shows a positive correlation in relation to DAG levels in cells up- or down-regulating GLTP synthesis (II – figure 4C). The observed changes in the DAG levels, as well as those observed in ceramide and SM, may be a response caused directly, or indirectly, by the alternation of the cellular GLTP activity, either through a disruption of the sphingolipid homeostasis or

through a possible interplay between GLTP and CERT. CERT, like GLTP, contains a FFAT-like motif which functions to direct the protein to the ER-compartment by binding to the ER-transmembrane VAPs [300]. Changes in cellular ceramide and SM, as well as cholesterol, is not only sensed by CERT, but also by OSBP [311]. OSBP, CERT and Nir2 are all proteins that contain the FFAT-like motif and are involved in maintaining DAG levels in the Golgi [246, 302, 332]. Similarly, GLTP might be involved in the orchestration of GSL homeostasis, synergistically, acting in concert with FAPP2 and the other FFAT-like motif containing LTPs.

Changes in the PC or PE levels, based on the MS analysis, did not correspond well with the radiolabeling experiments (II – figure 2, 5B and 6A). The radiolabeling experiments show a significant decrease in both PC and PE levels in cells with down-regulated GLTP, whereas up-regulation of GLTP expression did not show a significant difference. The MS analysis shows less dramatic changes in the overall PC and PE levels, regardless of treatment. Such variations might be attributed to the inherent differences between the two analysis methods. Based on the MS analysis, cells overexpressing GLTP show a significant decrease in PS levels, as well as a marked increase in CE levels (II – figure 6B and 4B). Interestingly, the degree of unsaturation in PS was also clearly decreased in these cells (II – figure 7D). In mammals, PS is synthesized in the ER, where a base-exchange reaction that is catalysed by PS synthase-1 (PSS-1) primarily uses PC as a substrate for serine exchange and subsequent PS generation [333]. PS synthase-2 (PSS-2) mainly uses PE as a substrate. PSS-1 and PSS-2 are both integral ER membrane proteins that localize to mitochondria-associated endoplasmic-reticulum subcompartments (MAMs), i.e. regions of the ER that closely associate with mitochondria. The enzymatic activities required for the synthesis of CE and free cholesterol have also been located in the MAM fractions [185]. In addition, GlcCer synthesis has been reported to occur, not only in the *cis*-Golgi [177–182], but also in the MAMs [185]. Furthermore, it has been suggested that PS might serve as the serine donor in the initial step in the ceramide biosynthesis, where serine and palmitoyl coenzyme A are condensed by serine palmitoyl transferase [334]. Alteration of GLTP expression might therefore also impact the metabolism of these lipid species, considering the above-mentioned synthetic pathways and their interconnected status. It is difficult to speculate what the reason for the reduction in the degree of unsaturation in PS is, in cells overexpressing GLTP. It is possible that these alterations occur in an effort to compensate for changes in the membrane packing and fluidity that the increased GLTP levels perhaps impart on the cell membranes.

5.1.8 Summarizing notes (II)

The cellular lipid metabolism is an intricately complex, interconnected system, and the analysis of even a small portion of such a system can be a harrowingly difficult task. Overall, while many of the observations presented here are quite difficult to interpret, and therefore also difficult to connect to possible GLTP activity, the results still offer valuable insights into GLTP and its possible cellular function. To summarize, the changes observed are likely to be a consequence of GLTP's involvement in binding, sensing or transporting GSLs in cells, while working together with the other VAP-binding LTPs. The changes regarding Gb3 synthesis are of particular interest and suggest that there may be a direct interplay between GLTP and FAPP2. Analysis of such synergistic functions, between GLTP and different LTPs, are therefore of high importance for future projects. It is quite probable that the various LTPs act in concert to regulate the overall lipid homeostasis in cells.

5.2 Metabolic conversion of ceramides in cells – a solvent-free delivery approach (III)

The HPTLC and MS analysis in publication II presents us with data that demonstrated changes, not only in the total masses of some of the different lipid species examined, but also in some of the chain-specific lipids. The ceramide data, for example, shows a decrease in the d18:0 ceramides as a result of GLTP up-regulation, whereas the d18:1 ceramides were overall less affected. This was further reflected by a similar decrease in the d18:0 SM levels. Similarly, the data show that some GSLs, of certain acyl-chain compositions, were affected by the up- or down-regulation of GLTP more strongly than others (II – figure 3A – 3D and 4A). Analysis of the more chain-specific changes in the cellular lipidome adds a significant level of complexity to the issue, but also raises an interesting point regarding GLTP and its possible functions. GLTP may have a preference when interacting with GSLs of varying acyl-chain compositions. As such, it is also plausible that GLTP may have some interaction with the ceramide synthesizing enzymes, subsequently affecting which ceramides are being produced. In mammals, ceramides are synthesized by six distinct ceramide synthases (CerS1-6). Each synthase is responsible for the production of ceramides of particular chain lengths [111, 112]. More than 100 distinct, naturally occurring ceramides have been identified using current LC-MS/MS technology [145]. Additionally, emerging evidence suggests that different ceramides, in terms of the length of their N-acyl chains, may be destined to perform different cellular functions (see section 2.5.3).

The ability to alter how cells produce different chain length sphingolipids would be very useful for elucidating whether GLTP, *in vivo*, shows any preference for sphingolipids of particular chain lengths or compositions. Although a labour-intensive process, genetic up- or down-regulation of the different CerSs is certainly a plausible approach for performing such a task. Individual up- and down-regulation of the various CerSs has been performed previously, demonstrating complex levels of interregulation and substitutive capabilities between the enzymes [335]. However, the simultaneous genetic alteration of more than one CerS rapidly increases the complexity of the matter. Alternative methodological approaches, for analysing what roles the different chain length ceramides play in cells, would therefore be welcome. Natural ceramides are molecules that exhibit very poor solubility water, and therefore cannot be effectively introduced to cells by dispersion into aqueous growth media. As a consequence, studies where ceramides have been introduced to cultured cells have primarily made use of DMSO or ethanol dispersions of the more water-soluble, non-physiological, short-chain ceramide variants [21, 336, 337]. Such studies have helped establish the ceramides as key players in the cellular apoptotic pathways, as well as several other cellular processes [145]. The short-chain ceramides, and the effects they impart on cells, may not fully reflect those of their endogenous counterparts. The role of endogenous ceramide has previously been investigated through the manipulation of the various enzymes involved in ceramide metabolism, such as the SMases [338, 339]. Even so, a method for introducing longer-chain, physiological ceramides to cells would be very useful in elucidating the possible roles of the different ceramide species more precisely.

Lönnfors et al. previously showed that C6- and C16-ceramide (**figure 17**) can form stable bilayers with cholesteryl phosphocholine (CholPC), in an equimolar ratio [312]. Ceramide and cholesterol do not react favourably in the absence of phospholipids with large polar headgroups, however, when ceramide is complexed with CholPC, the phosphocholine headgroup acts to protect the ceramide from the aqueous environment and allows for the formation of lipid bilayer vesicles. Similarly, CholPC can form complexes with cholesterol and dimyristoglycerol [312]. Furthermore, Sukumaran and co-workers demonstrated that complexes of CholPC and C6-ceramide could be used to deliver ceramide to cells in culture. Using this delivery method, the inhibition of cell proliferation was reportedly more drastic, when compared to formulations of C6-ceramide and DMSO [340]. In publication III, we set out to expand upon this methodology and to test whether longer-chain ceramides could successfully be introduced to cells in CholPC complexes.

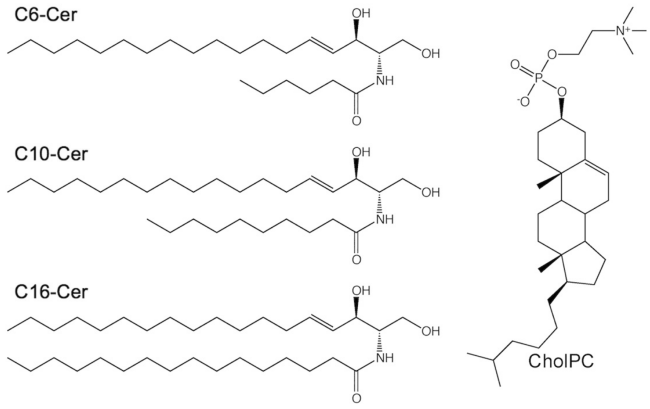

Figure 17. Molecular structures of the different chain length ceramides and CholPC used in this study. Figure is from publication III.

5.2.1 The rate of ceramide uptake depends on the acyl chain length (III)

Initially, we tested whether cells were able to take up C10- and C16-ceramide from the growth medium, when the ceramides were complexed with CholPC in an equimolar ratio. We compared the rates of radiolabel uptake, to that of the already established uptake of C6-ceramide from similar complexes [340]. In the experiments described below, the radionuclide resided on the sphingosine base of the ceramide, unless otherwise stated. We found that all three ceramide/CholPC complexes demonstrated successful ceramide delivery to HeLa cells, when the cells were exposed to growth medium containing 50 μM of the respective ceramides in complex (**figure 18A**).

C6-ceramide had the highest uptake rate, followed by C10-ceramide and C16-ceramide. C10-ceramide demonstrated an initial uptake rate of roughly 50% to that of C6-ceramide, whereas C16-ceramide similarly only demonstrated a 5% uptake rate. For C6- and C10-ceramide treated cells, radiolabel uptake seemed to plateau within 6 hours from the beginning of the experiment. To test for equipotent delivery, ceramide uptake was analysed using two different concentrations for each ceramide. Radiolabel uptake seemed to directly correlate with the ceramide concentration in the growth medium, demonstrating an equipotent delivery, at least within the concentrations and timeframe used (**figure 18B**).

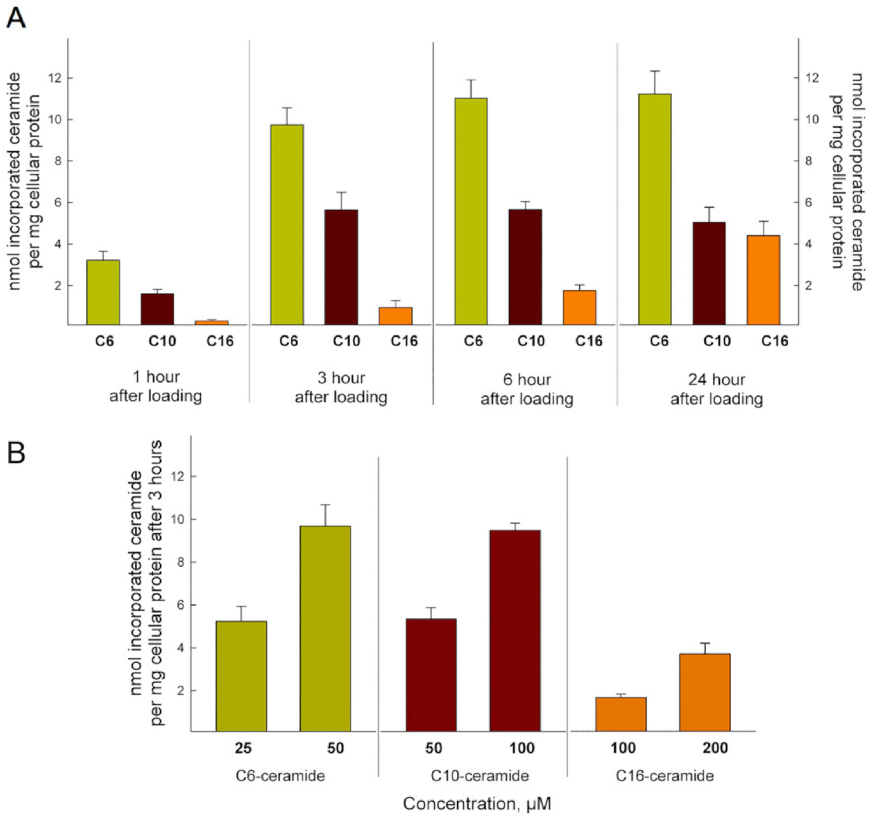

Figure 18. **A**) The amounts of radiolabeled C6-, C10- and C16-ceramide uptake as nmol/mg cellular protein, as a function of time. HeLa cells were labeled with radioactive ceramide (50 µM), with the radionuclide in positions 4 and 5 of the sphingosine backbone. The uptake was analysed by HPTLC and scintillation counting. **B**) Amounts of ceramide precursor incorporation into HeLa cells at different loading concentrations of ceramides after a 3-hour incubation. Results are means +/- SEM of at least 3 independent experiments. Figure is from publication III.

5.2.2 Ceramide metabolism depends on the ceramide uptake rate (III)

Once the cellular uptake had been established, we decided to take a closer look at how the different ceramides were metabolised by the cells. Cells were treated with 50 µM of each respective ceramide for 3, 6 and 24 hours, whereafter total lipids were extracted from the cells and the radiolabel distribution was analysed. The results show that the incorporated ceramides

are metabolised and that the radiolabel is distributed into other sphingolipids as a function of time (III – figure 3). It is interesting to note that in the C6-ceramide treated cells, a large portion of the radioactivity could be found in the simple GSLs (i.e. the pooled GlcCer, GalCer and Glc/GalCer-OH), at the 24-hour mark (III – figure 3A). In contrast, in both the C10- and C16-ceramide treated cells, most of the radioactive signal could be found in SM, after 24 hours of treatment (III – figure 3B and 3C). Chapman et al. previously showed that cancerous cells in culture convert C6-ceramide to either SM or GlcCer, in a manner depending on the initial concentration of ceramide used in the growth medium [341]. In lower-dose treatments, cancer cells seem to favour conversion of C6-ceramide to SM, whereas higher-dose treatments result in conversion to GlcCer. Glycosylation may perhaps be a general survival mechanism, by which cells dispose of unnaturally high amounts of ceramide. Indeed, it has been previously shown that when ceramides are co-administered with GlcCer synthase inhibitors, as well as P-glycoprotein antagonists, ceramide-induced apoptosis increases [341–343]. Presumably, as glycosylation is inhibited, the higher accumulation of ceramide leads to a more drastic anti-proliferative effect.

Since, in our experiments, the various ceramides demonstrated different rates of cellular uptake (**figure 18**), we next decided to test whether metabolic conversion, similar to what observable in the C6-ceramide treated cells (III – figure 3A), was attainable with the longer-chain C10- and C16-ceramides. By increasing the concentrations of the C10- and C16-ceramide complexes in the culture medium, we hoped to simulate the effects observed in the C6-ceramide experiments. HeLa cells were exposed to varying concentrations of the respective ceramides for 24 hours, whereafter the radiolabel distribution into the cellular lipids was analysed as described above. This time, however, a more in-depth analysis of the radiolabel distribution was performed (**figure 19** and **20**). Suitable ceramide concentrations were estimated based on the incoporation rates in **figure 18**, and from previous works in the literature [341].

In these experiments, lower-dose treatment with C6-ceramide (1 μM) resulted in most of the radioactive signal to be found in SM, whereas the higher-dose treatments (25 – 100 μM) demonstrated strong signals in C6-GlcCer (**figure 19A**). Similar results were observed for the C10-ceramide treated cells (**figure 19B**), where a clear glycosylation of C10-ceramide was observable in cells treated with 100 μM of the lipid. Interestingly, glycosylation was not as pronounced in cells treated with 200 μM of C10-ceramide.

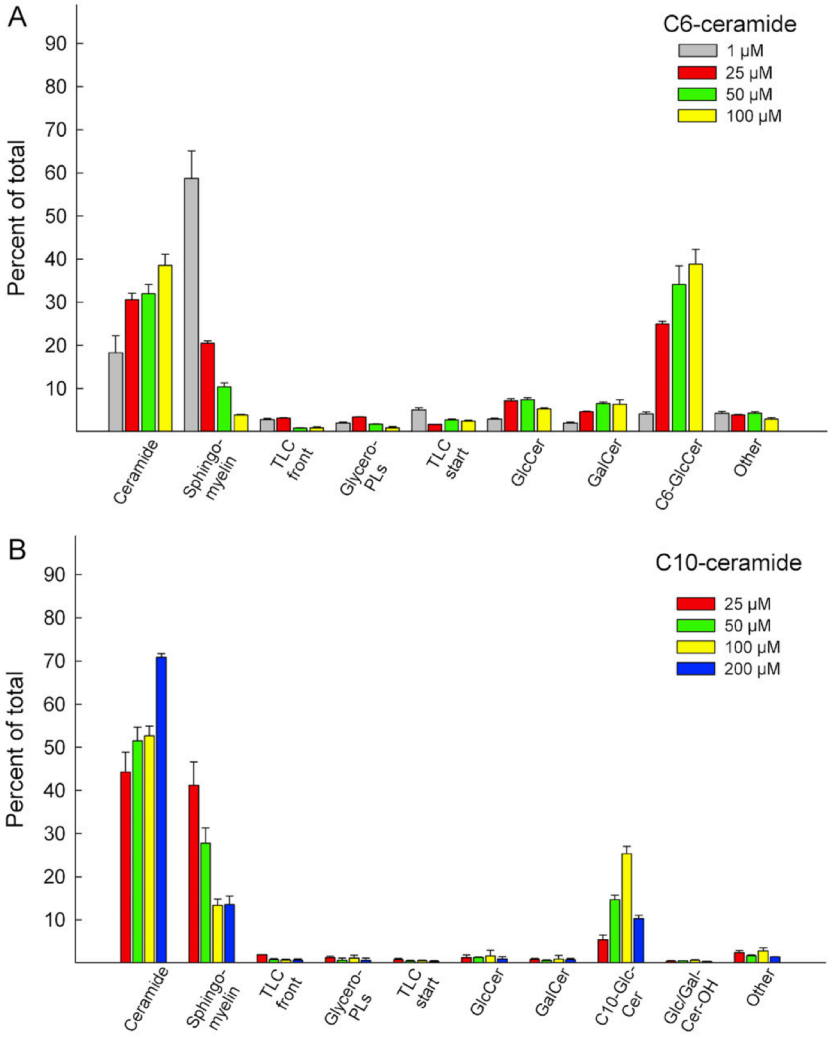

Figure 19. Metabolic conversion of (**A**) C6- and (**B**) C10-ceramide by HeLa cells at various concentrations. The ceramides were [^{3}H]-labeled in the sphingosine. After 24 hours of ceramide uptake the total lipids were extracted and analysed by HPTLC and scintillation counting. "Start" and "Front" refer to the labeled lipids remaining on the application spot, or being eluted along with the solvent front, "Other" refers to the traces of radiolabeled lipids between the identified spots on the HPTLC plate (see III – supplemental figure 1). Results are means +/- SEM of at least 3 independent experiments. Figure is from publication III.

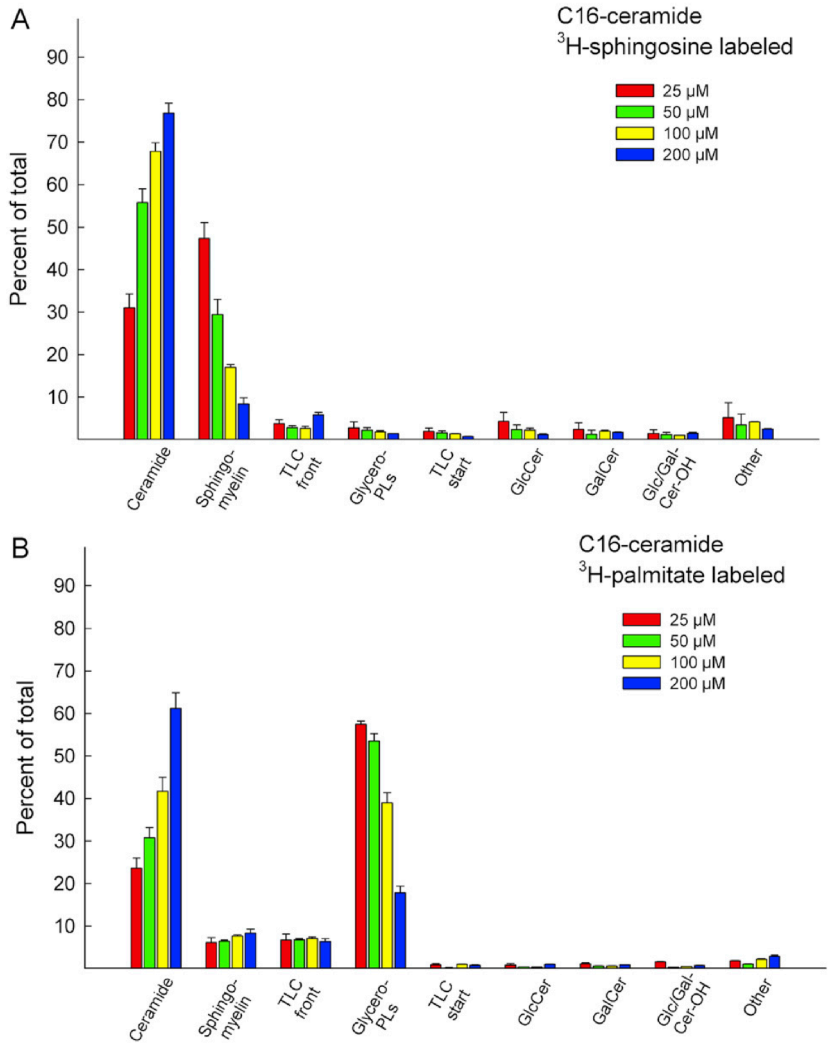

Figure 20. Metabolic conversion of C16-ceramide at various concentrations. The C16-ceramide was either [^{3}H]-labeled in the sphingosine backbone (**A**) or in the palmitic acid portion (**B**). After 24 hours of treatment, the total lipids were extracted and analysed by HPTLC and scintillation counting. "Start" and "Front" refer to the labeled lipids remaining on the application spot, or being eluted along the solvent front, "Other" refers to the traces of radiolabeled lipids between the identified spots on the HPTLC plate (see III – supplemental figure 1). Results are means +/- SEM of at least 3 independent experiments. Figure is from publication III.

For C16-ceramide, both a sphingosine ^{3}H-labeled and a palmitic acid ^{3}H-labeled ceramide was used, in separate experiments (**figure 20**). In these experiments, higher-dose (100 – 200 μM) treatments with C16-ceramide did not show a marked increase in GSL radioactivity. Instead, large portions of the radioactive signal remained as ceramide. This was evident for both the sphingosine ^{3}H-labeled and the palmitic acid ^{3}H-labeled C16-ceramide. In the experiments where the radiolabel resided on the acyl chain of the ceramide, a significant portion of the radioactivity was distributed to the glycerophospholipids, especially in the lower-dose experiments (**figure 20B**). This suggests that the externally introduced C16-ceramide is subject to significant degradation, at least under the conditions used in publication III.

5.2.3 Externally introduced C16-ceramide is mainly degraded (III)

In an attempt to clarify how C16-ceramide is metabolised, we investigated whether inhibition of ceramide degradation using the lysosomal inhibitor chloroquine [344], as well as the ceramidase inhibitor ceranib-2 [345], could be used to inhibit the supposed degradation of the introduced C16-ceramide. When the palmitic acid ^{3}H-labeled C16-ceramide (50 μM) was introduced to cells in the presence of these substrates, a substantial reduction in glycerophospholipid radioactivity could be observed (III – figure S2). Based on these observations, it seems likely that the signal observed in SM in **figure 20A** would similarly be due to the recycling of the sphingosine base, as a result of ceramide degradation. This assumption was further put to the test, when we investigated whether the ceramide synthase inhibitor fumonisin B1 [346] could be used to inhibit the incorporation of the putatively liberated sphingosine into SM (III – figure S3). The results show that a significant reduction in SM radioactivity is achieved when cells are simultaneously treated with 200 μM fumonisin B1 and 50 μM sphingosine ^{3}H-labeled C16-ceramide. These results support the assumption that C16-ceramide is mainly degraded when introduced to cells in the manners described, and that this degradation, at least partly, takes place in the lysosome. However, neither of the inhibitors resulted in complete suppression of ceramide degradation and one cannot therefore rule out that some of the signal observed in SM (and the GSLs) came from directly modified ceramide. In fact, the act of using inhibitors may in itself result in the cell up-regulating the use of the externally delivered ceramides for direct synthesis of higher sphingolipids. Indeed, the chloroquine-mediated inhibition of lysosomal degradation also led to a increase in SM radiolabeling, suggesting that the C16-ceramide may have more readily been used for direct SM synthesis (III – figure S2).

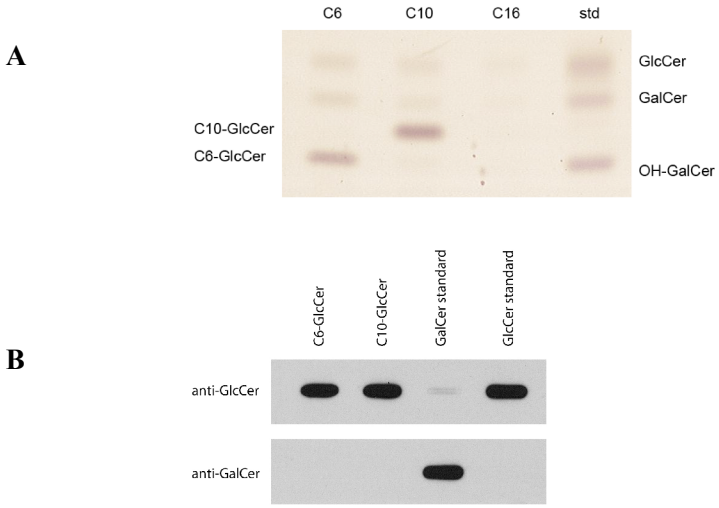

Figure 21. **A**) A representative HPTLC plate analysis of lipid extracts from HeLa cells treated with high doses of ceramide (50 μM C6-ceramide, 100 μM C10-ceramide and 200 μM C16-ceramide). The image illustrates the separation of GlcCer with incorporated chain specific ceramides (C6 or C10) from endogenous GSLs. The plate was stained with orcinol to detect the carbohydrate groups of the GSLs. **B**) A dot blot analysis was performed to verify that the lipid spots observed in the high-dose C6- and C10-ceramide treatments were GlcCer. The C6- and C10-GlcCer spots were scraped off an iodine stained HPTLC plate, extracted from the silica and analysed using anti-GlcCer and anti-GalCer antibodies. Figure is adapted from publication III.

Whether or not a similar recycling of the ^{3}H-labeled sphingosine base was observed in the C6- and C10-ceramide treated cells, is not discernible from the experiments presented in publication III. However, since endogenous GlcCer and GalCer also showed a marked increase in the high-dose C6-ceramide treated cells (**figure 19A**), it can be presumed that at least some recycling takes place. This assumption is supported by previous research, where it was shown that C6-ceramide loading of A549 adenocarcinoma epithelial cells leads to higher synthesis of endogenous ceramide, due to recycling of the sphingosine backbone, but not due to elongation of the short acyl chain [347]. It is interesting to note that high-dose treatment with C6- and C10-ceramide shows an accumulation of radioactivity in C6- and C10-GlcCer. Neither LacCer (contained within the "other" spot), to which GlcCer is a direct synthetic precursor, nor any of the higher GSLs (contained within

the "TLC start" spot) showed any marked increases in radioactivity in response to the treatments (**figure 19A** and **19B**). This suggests that C6- and C10-GlcCer are not further glycosylated to form higher GSLs. It is worth noting that in the respective high-dose treatment experiments, the C6-GlcCer and C10-GlcCer spots were visually clearly discernible from the endogenous GlcCer and GalCer on orcinol-stained HPTLC plates, whereas C16-GlcCer was not (**figure 21A**). It can, therefore, be assumed that the radioactivity present in the C6-GlcCer and C10-GlcCer spots accurately represents GlcCer that has been directly glycosylated from C6- and C10-ceramide, respectively. To verify the lipid identity of these spots, C6-GlcCer and C10-GlcCer were validated by immunoblotting, using rabbit anti-GlcCer and anti-GalCer antibodies (**figure 21B**).

5.2.4 The different ceramides exhibit varying degrees of anti-proliferative effects (III)

The apoptotic effects that ceramides have on cells are evident. External apoptotic stimuli have been found to increase ceramide levels in cells and exogenous treatment with short-chain ceramide has been shown to cause cells to undergo apoptosis [21, 23, 24, 348]. Ceramide has been implicated in both the extrinsic and the intrinsic apoptotic signaling pathways. The methods of action include inhibition of the PI 3-kinase/Akt pathway as well as the permeabilization of the mitochondrial outer membranes [129, 130, 349–352].

As previously reported, cells treaded with C6-ceramide that had been complexed to CholPC in vesicles, demonstrated more dramatic apoptotic effects and inhibition of cell growth, when compared to cells treated with DMSO-solubilized ceramide [340]. In publication III, the respective lower-dose ceramide treatments did not result in noticeable cell-detachment, whereas higher-dose treatments did. Therefore, to establish the degree of inhibition the different complexes had on cell proliferation, we performed a resazurin-conversion assay on cells treated with increasing concentrations (25 – 200 μM, 24 hours) of the various ceramides and respective cholesterol-CholPC controls. Resazurin is a redox indicator that yields a colorimetric change and a fluorescent signal in response to cellular metabolic activity, when added to cells in culture [353]. A decrease in these signals can be directly correlated with loss of cell viability. The cell viability in each sample was normalized to PBS-vehicle treated controls (**figure 22**). C6-ceramide was the most potent in decreasing cell viability, followed by C10- and C16-ceramide. This was perhaps not unexpected, considering the rates of cellular uptake that the different ceramides previously demonstrated (**figure 18**).

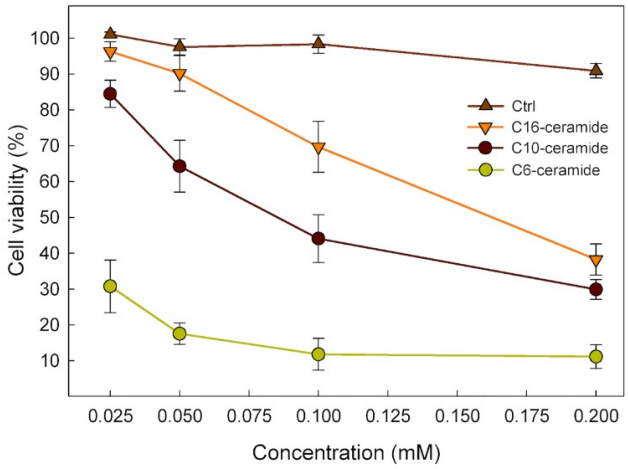

Figure 22. Cytotoxic effects of the various ceramides. HeLa cells were treated with increasing concentrations of C6-, C10-, C16-ceramide or cholesterol, complexed with CholPC, for 22 h and compared to vehicle (PBS) treated cells. Subsequently, a resazurin reduction assay was performed. Viability of the HeLa cells is represented as percentage of survival compared to the PBS control. Results are means +/- SEM of at least 3 independent experiments. Figure is from publication III.

Based on these results, it seems as if C6-ceramide has an intrinsically stronger anti-proliferative effect, when compared to C10-ceramide. C6-ceramide demonstrated more efficient inhibition of cell viability, even when the molar uptake of C10-ceramide was higher than that of C6-ceramide (25 µM C6-ceramide treatment vs. 100 µM C10-ceramide treatment, **figure 18B** and **22**). Furthermore, it is interesting to note that treatment with 200 µM C16-ceramide led to a comparable inhibition of cell viability to that of a 100 µM treatment with C10-ceramide (**figure 22**), even though the initial rate of C16-ceramide incorporation at these conditions was less than half of that of C10-ceramide (**figure 18B**). One explanation to this phenomenon may lie in how the cells metabolise, or rather, how the cells *do not* metabolise C16-ceramide, when the lipid is presented at high concentrations. As the concentration of C16-ceramide in the culture medium was increased, more of the radioactive signal remained as ceramide, presumably due to a decrease in the degradation and the subsequent recycling of the radiolabel (**figure 20**). As a result, the increased cellular ceramide concentration might have lead to stronger anti-proliferative effects. Alternatively, while a high-dose treatment with cholesterol and CholPC (200 µM) did not affect cell viability to a significant

degree, one cannot exclude the possibility that simultaneously high levels of ceramide and CholPC may result in combinational effects, which alter how the ceramides are metabolised in cells. It is possible that such a combinational effect might result in the inhibition of ceramide glycosylation and/or degradation. As noted previously, when C10-ceramide was complexed with CholPC and presented to cells at a concentration of 200 μM, subsequent glycosylation to C10-GlcCer was significantly decreased, when compared to the corresponding 100 μM treatment (**figure 19B**). The literature does describe similar inhibitory effects on sphingolipid synthesis, which can be attributed to increased cholesterol levels in cells. The loading of HSF cells with cholesterol, with the help of cyclodextrin-inclusion complexes, has previously been shown to decrease the *de novo* synthesis of both ceramide and SM [354]. However, since CholPC is structurally different from naturally occurring cholesterol, the results in publication III are not necessarily comparable, and parallels should be drawn with caution.

5.2.5 Summarizing notes (III)

Overall, the results from publication III present us with a valid, solvent-free method of introducing ceramides to cultured cells. The ceramides are taken up at rates that correspond with their acyl chain lengths, where shorter lipids are internalized more quickly than their longer-chain counterparts. C6-ceramide seems to have an intrinsically stronger anti-proliferative effect when compared to C10-ceramide. Higher-dose treatment of HeLa cells with C6- and C10-ceramide resulted in glycosylation of the ceramide, which produced corresponding chain length GlcCer. C16-ceramide was mainly degraded when introduced to cells at lower doses and its metabolisation was reduced at higher doses. Since the degree of ceramide glycosylation seems to be linked to the rate of ceramide uptake, the overall slower incorporation speed of the C16-ceramide was perhaps not enough to trigger a noticeable glycosylation. Whether or not similar glycosylation of longer-chain ceramides is attainable at sufficient internalization rates is something that warrants further exploration.

5.4 Future prospects (I, II and III)

From publications I and II, it is fairly evident that GLTP is biologically connected to the GSLs. Furthermore, the discovery that GLTP expression correlates with levels of cellular GlcCer and Gb3, opens up interesting viewpoints regarding the protein's function. As previously mentioned, these results raise the possibility for the existence of a cellular connection between

GLTP and FAPP2. Down-regulation of FAPP2 leads to an accumulation of GlcCer that is comparable to the decrease in the levels of LacCer and Gb3 in cells [184, 249], whereas GLTP knockdown does not significantly alter GlcCer synthesis (**figure 15**, **16A** and [256]). Furthermore, the ablation of FAPP2 has a more dramatic effect on Gb3 levels, when compared to GLTP knockdown (~50% reduction vs. ~25% reduction, respectively). Taken together, these results suggest that GLTP and FAPP2 carry out distinct functions regarding Gb3 synthesis, although one should be mindful of the possibility that the observed differences in Gb3 reduction may be due to methodological variations. There is evidence suggesting that FAPP2 acts as a *bona fide* transporter of GlcCer in cells [183, 249], whereas the results presented here, and in previous works, may be more indicative of a sensory role for GLTP [256]. It is also clear that neither GLTP nor FAPP2 is indispensable when it comes to Gb3 synthesis, suggesting that the proteins may compensate for the lack of each other. If (and how) the two proteins come together to regulate Gb3 synthesis is clearly something that warrants further research. While the generation of double knockout GLTP- and FAPP2-mutants has previously been unsuccessful [184], unpublished results from our laboratory demonstrate that transient down-regulation of both proteins simultaneously in cultured cells is indeed possible. Consequently, this allows for the analysis of whether or not GLTP and FAPP2 are involved in compensatory functions, and possibly whether these proteins interact in cells. Furthermore, the utilization of fluorescence microscopy-based analysis, such as bimolecular fluorescence complementation (BiFC) [355], could demonstrate the plausibility of a GLTP-FAPP2 *in vivo* interaction. Indeed, we have recently demonstrated that cellular interaction between the ER-residing VAP-A and GLTP occurs, using BiFC-microscopy (unpublished results).

The MS results from publication II suggest that GLTP might be involved in the synthesis of lipids of particular chain lengths and compositions. However, these details are difficult to interpret, since modulation of GLTP expression seemingly affects the prevalence of certain acyl chain compositions, not just in the GSLs, but in non-sphingolipids as well. Nevertheless, of the analysed chain-specific Gb3s, all species positively correlate with GLTP expression (**figure 16B**), with the biggest difference occurring in the 18:0 and 24:1 acyl-chain Gb3 species, in cells overexpressing GLTP. These particular Gb3 species might function as a good starting point for further experiments, if one were to hypothesize that GLTP is somehow involved in the cellular regulation of GSLs of particular acyl-chain compositions. The selective alteration of the expression of the various CerSs could possibly be a good method for elucidating this putative GLTP-lipid specificity. Subsequently, adapting the

methodology presented in publication III for similar analysis could likewise be plausible. At the present, study of the chain-specific binding and transfer of GSLs by GLTP is already being carried out in our laboratory, using SPR- and FRET-based *in vitro* assays.

The method by which ceramide is internalized from the CholPC complexes is unknown. Sukumaran and coworkers previously suggested that C6-ceramide uptake mainly takes place by monomer exchange between the complexes and the PM [340]. Indeed, since the ceramide/CholPC vesicles likely need to exist in equimolar complexes for maximal stability, some sort of lipid replacement must occur, as the ceramides are internalized. No lipid precipitate was observable in the reaction medium in any of the experiments performed in publication III, as evidenced by routine microscopy analysis, suggesting that little or no complex collapse occurred due to shifts in vesicular CholPC-to-lipid molar ratios. Since the longer-chain ceramides in publication III are taken up at markedly diminished rates, and since the acyl-chain length of lipids have been shown to correlate with their transfer efficiency between membranes [356, 357], monomer exchange presumably remains the most likely scenario of ceramide uptake. However, some degree of endocytic internalization cannot be ruled out, as it was previously observed that CholPC was also internalized from the complexes (albeit to a minor degree) [340]. By extension, it would be interesting to study what kind of lipids the CholPC complexes putatively replace in the membrane as the ceramides are taken up by the cells. Subsequently, the method might be adapted as a simple tool for analysing the ceramide and/or cholesterol content in the PM of cultured cells.

GLTP expression has previously been linked to the prevalence of short-chain ceramide in cells [358]. Zou and coworkers showed that when HeLa cells were incubated with C6-ceramide, an Sp1/Sp3 transcription factor-mediated activation of the GLTP promoter was induced. Interestingly enough, short-chain C8-GlcCer did not result in similar results. The authors hypothesized that the externally introduced ceramide would subsequently result in an increased GLTP promoter activation in one of two ways: either by altering Sp1/Sp3 binding affinity or by altering Sp3 acetylation status. A ceramide-induced acetylation of Sp3 has previously been shown to regulate the transcriptional activity of human telomerase reverse transcriptase [359]. Furthermore, the promoter activity of GlcCerS has also been shown to increase in response to ceramide treatment [360], which is in agreement with the results shown in publication III, regarding the increased endogenous GlcCer synthesis as well as the glycosylation of C6-ceramide (**figure 19A**). Preliminary experiments from our laboratory show that GLTP levels are

indeed affected by the introduction of short-chain C6-ceramides, however, the results seem somewhat contradictory to those which were reported by Zou and coworkers [358]. Our results show that both C6-ceramide and C8-GlcCer result in a clear *reduction* of endogenous GLTP levels within 24 hours of treatment (**figure 23**).

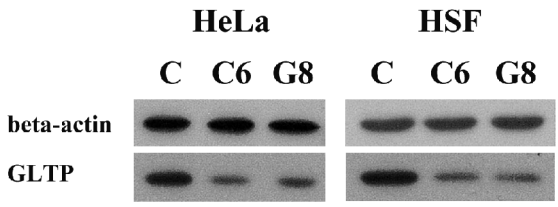

Figure 23. GLTP levels in HeLa (left) and HSF (right) cells treated with DMSO-solubilized C6-ceramide (C6, 25 μM) or C8-GlcCer (G8, 30 μM) for 24 hours, as determined by Western blotting. C = untreated vehicle controls. Beta-actin was used as a loading control. Unpublished results.

It should be noted, however, that mRNA expression does not necessarily correlate positively with the total corresponding protein levels in cells. Additionally, Zou and co-workers did not analyse GLTP mRNA or promoter activity past 12 hours of ceramide treatment, whereas the results presented in **figure 23** are from a 24-hour treatment. It is interesting to speculate why GLTP levels would be reduced as a result of these treatments. The effect is likely to be linked to the glycosylation events observed in these cells, as both C6-ceramide and C8-GlcCer result in a similar reduction of GLTP levels. As mentioned in section 5.2.3, very little of the glycosylated short-chain ceramide is further metabolised to produce higher GSLs. Therefore, there must be an accumulation of short-chain GlcCer at some compartment in the cell. This accumulation may act to "block" GLTP from interacting with natural GlcCer, subsequently leading to a decreased production (or perhaps to an increased degradation) of GLTP. It is possible that the unnatural chain length GlcCer cannot be sensed or transported by GLTP *in vivo*, perhaps due to improper packing into membranes or due to its intrinsically more rapid membrane lateral movement. In the future, it would be interesting to test what effects longer-chain ceramides might have on GLTP, by adapting the method from publication III to deliver natural ceramides to cells, at rates sufficient enough to putatively induce glycosylation. In its present form, however, the

method may be not be suitable for this purpose. As the chain length of the complexed ceramide is increased, the subsequent rate of uptake decreases. While this can, to some extent, be corrected for by simply adding more of the complexed ceramide to the growth medium, there is presumably a limit to how high one can go. As the results from publication III suggest, cells may be subjected to unexpected combinational effects, arising from simultaneously high levels of ceramide and CholPC. Therefore, the method may have to be developed and studied more extensively, if one were to use it for analysis using longer-chain ceramides (e.g. 24:1). One possible way by which an increased uptake might be achieved is the simultaneous addition of a lipid-transferring substrate to the growth medium, such as purified, recombinant CERT. Similar experiments have already demonstrated functionality; recombinant GLTP has been shown to successfully extract GSLs from the PM of cells [184], and could presumably likewise be used to transfer GSLs from donor membranes to cultured cells [361].

6. CONCLUSIONS

As a whole, the work described in this thesis explores the metabolism and function of sphingolipids. In particular, their relationship to GLTP is examined, in an effort to uncover hints toward the protein's cellular function.

Overall, the previous postulations regarding GLTP's function are strengthened. GLTP has been suggested to function as a carrier or sensor of GSLs, based on structural analysis, as well as model and cellular experiments. In our work, we show that GLTP expression correlates positively with changes in cellular GSL levels, particularly with GlcCer. More specifically, GLTP expression seems to correlate with the levels of *de novo* synthesized GlcCer, suggesting that GLTP might perform a function at, or close to, the site of GlcCer synthesis. Furthermore, the finding that Gb3 synthesis is affected by changes in cellular GLTP levels suggests that GLTP may have a similar function to FAPP2, which is likewise involved in the synthesis of Gb3. By extension, it is plausible that GLTP and FAPP2 might interact and work together to maintain the cellular GSL homeostasis. This interaction could putatively take place at the ER, where both proteins perhaps interact with the ER-resident membrane protein VAP-A, through their FFAT-like motives. The results also suggest that alteration of cellular GLTP expression induces changes in the levels of lipids of certain acyl-chain compositions. As such, it is possible that GLTP might be involved in cellular events that employ GSLs of specific acyl-chain compositions.

In publication III, we set out to examine a novel method of introducing various chain length ceramides to cells, in the hopes that this method could eventually be utilized in a more complete mapping of ceramide function. The results show that complexation with CholPC offers a viable method for solubilizing ceramides of various chain lengths in aqueous solutions and, furthermore, that the ceramides are taken up, and metabolised, when presented to cells in culture. Based on the results, C6-ceramide seems to have an intrinsically stronger anti-proliferative effect when compared to C10-ceramide. Interestingly, it was also observed that when short-chain ceramides were introduced to cells at sufficient rates, their metabolism shifted towards GlcCer synthesis. We were not able to observe similar conversion of C16-ceramide, possibly due to the significantly slower uptake of this lipid from the CholPC complexes. The reasons behind the glycosylation, and whether or not naturally occurring, longer-chain ceramides also undergo such conversion when presented to cells at sufficient rates, are interesting questions that should be addressed in future studies.

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